Université de Montréal

Studies on IL-18 in HIV infection: Effects of the cytokine on intestinal integrity, and platelets as a new source of the cytokine

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Résumé

L'interleukine IL-18 (IL-18), un membre de la famille de l'IL-1, est une cytokine pro-inflammatoire multifonctionnelle. Elle est produite par les monocytes, les macrophages, les cellules dendritiques, les cellules épithéliales, les kératinocytes et le cortex surrénal dans le corps humain. Cette cytokine est d'abord produite comme une protéine précurseure inactive, qui est par la suite clivée en une forme mature par la caspase-1 activée. La caspase, en elle-même, existe comme précurseur inactif dans les cellules humaines et requiert l'assemblage d'inflammasomes pour son activation. L'IL-18 pour joue un rôle clé dans la médiation des conditions inflammatoires. Notre laboratoire et d'autres ont montré que l'infection par le VIH est accompagnée d'une augmentation des taux circulants d'IL-18 avec une diminution des niveaux de son antagoniste, l'interleukine-18 binding protein (IL-18BP).

Dans cette thèse, nous démontrons pour que l'IL-18 est également produite et sécrétée par les plaquettes humaines lors de leur activation. Les plaquettes contiennent des composants de l'inflammasome. Ils assemblent et activent la caspase-1, qui ensuite traite le précurseur de l'IL-18 dans sa forme mature au cours du processus d'activation des plaquettes. La cytokine est synthétisée de novo lors de l'activation des plaquettes. Contrairement à l'IL-18, les plaquettes expriment constitutivement l'IL-18BP, et la libèrent de manière constitutive, ainsi que lors de l'activation. L'IL-18 et l'IL-18BP sont colocalisés avec CD63, un marqueur pour les granules α des plaquettes. L'IL-18 libéré des plaquettes constitue la source principale de cette cytokine dans la circulation humaine chez les individus sains. Nous avons identifié des concentrations faibles de cette cytokine dans les lysats de plaquettes chez les individus infectés par le VIH par rapport à ceux en santé. D'autre part, les concentrations ont été augmentées dans le sérum et le plasma pauvre en plaquettes chez les individus infectés. Des résultats similaires ont été obtenus avec l'IL-18BP dans les lysats de plaquettes d'individus sains et infectés par le VIH. Cependant, des quantités plus faibles de cet antagoniste ont été trouvées dans le sérum et le plasma pauvre en plaquettes d'individus infectés par le VIH par rapport à ceux en santé. Nos résultats ont des implications importantes pour les maladies inflammatoires chroniques dans laquelle une activité accrue de l'IL-18 joue un rôle pathogène.

Le VIH est également accompagné par une inflammation intestinale et une diminution de l'intégrité intestinale, mesurée par la réparation de la muqueuse, la régénération et la perméabilité. Cependant, on en sait peu sur la relation entre le niveau élevé de l'IL-18 associé à l'infection au VIH et la perméabilité intestinale: ceci n'a jamais été étudié. Dans cette thèse, nous démontrons le rôle du virus et sa protéine Tat à augmenter la production d'IL-18 chez deux lignées de cellules épithéliales intestinales (HT29 et Caco2) ainsi qu'une diminution de l'IL-18BP. L'IL-18 induit une hyperperméabilité de la barrière épithéliale en perturbant à la fois les jonctions serrées et adhérentes, et ce, en modulant l'expression et la distribution de l'occludine, de claudine-2 et de la bêta-caténine. Une désorganisation de l'actine F a également été observée dans les cellules lors de l'incubation avec l'IL-18. Les mêmes observations ont été faites avec la protéine Tat du VIH-1. Après une incubation prolongée, l'IL-18 a causé la mort des cellules intestinales et induit l'apoptose par l'activation de la caspase-1 et la caspase-3. Fait intéressant, les taux plasmatiques de lipopolysaccharides chez trois catégories différentes de patients au VIH (ART-naïf, ART-traitée et contrôleurs élite) sont en corrélation avec les niveaux plasmatiques de l'IL-18. Enfin, nous avons étudié la voie de signalisation à travers laquelle l'IL-18 induit une perméabilité intestinale accrue.

En bref, nos études identifient les plaquettes comme une source importante d'IL-18, et leur activation lors d'une infection à VIH contribue à des concentrations accrues de cette cytokine. Le virus entraine également l'augmentation de la production de cytokines par les cellules épithéliales intestinales. L'activité biologique accrue de ces cytokines contribue à la pathogenèse du sida en augmentant la perméabilité intestinale et en causant la mort des cellules intestinales. L'IL-18 pourrait servir de cible moléculaire pour retarder la progression du sida et réduire l'inflammation chronique dans un stade précoce d'une infection à VIH.

Mots-clés: Interleukine IL-18 (IL-18), protéine de liaison IL-18 (IL-18BP), VIH, plaquettes, perméabilité intestinale.

Abstract

Interleukin IL-18 (IL-18), a member of the IL-1 family, is a multifunctional pro-inflammatory cytokine. It is known to be produced by monocytes, macrophages, dendritic cells, keratinocytes and the adrenal cortex in the human body. This cytokine is produced as an inactive precursor protein, which is cleaved into mature form by activated caspase-1. The caspase itself exists as an inactive precursor in human cells and requires inflammasomes assembly for its activation. IL-18 has been shown to play a key role in mediating different inflammatory conditions. Our laboratory and others have shown that HIV infection is accompanied with increased circulating levels of IL-18 along with decreased levels of its antagonist IL-18 Binding Protein (IL-18BP).

In this thesis, we show that IL-18 is also produced and secreted by human platelets upon activation. The platelets also contain components of the inflammasome. They assemble and activate caspase-1 and process the precursor IL-18 into its mature form during the platelet activation process. The cytokine is synthesized in platelets de novo upon activation. Contrary to IL-18, the platelets constitutively express pre-formed IL-18BP, and release it constitutively as well as upon activation. Both IL-18 and IL-18BP colocalized with CD63, a marker for the platelet α granules. Platelet-released IL-18 constitutes the main source of this cytokine in the human circulation in healthy individuals. We found decreased amounts of this cytokine in the platelet lysates in HIV-infected individuals as compared to the healthy ones. On the other hand, its concentrations were increased in the serum and platelet-poor plasma in infected individuals. Similar findings were obtained with IL-18BP in platelet lysates from healthy and HIV-infected individuals. However, lower amounts of this IL-18 antagonist were found in the serum and platelet-poor plasma from HIV-infected individuals compared with the healthy ones. Our findings have important implications for chronic inflammatory disease conditions in which increased IL-18 activities play a pathogenic role.

HIV is also accompanied by intestinal inflammation and decreased intestinal integrity as measured by mucosal repair, regeneration and permeability. However, little is known concerning the relation between high level of IL-18 associated to HIV infection and intestinal

permeability. In this thesis, we demonstrate the role of HIV and its protein Tat in increasing IL-18 production in two intestinal epithelial cell lines (HT29 and Caco2) and decreasing IL-18BP. IL-18 induces epithelial barrier hyperpermeability by disrupting both tight and adherens Junctions by modulating expression and distribution of occludin, claudin-2 and beta-catenin. Disorganization of F-actin was also observed within the cells upon incubation with treated by IL-18. Upon prolonged incubation, IL-18 caused intestinal cells death and induced apoptosis by activating caspase-1 and caspase-3. Interestingly, the plasma levels of lipopolysaccharide in three different categories of HIV-infected patients (ART-naïve, ART-treated and Elite controllers) correlated with their IL-18 plasma levels. Finally we investigated the signaling pathway through which IL-18 induces increased intestinal permeability.

Briefly, our studies identified platelets as an important source of IL-18, and their activation in HIV infection contributes to enhanced concentrations of the cytokine. The virus also induces increased production of the cytokine from intestinal epithelial cells. Increased biological activities of the cytokine contribute towards AIDS pathogenesis by increasing intestinal permeability and causing death of intestinal cells. The cytokine may serve as a molecular target for delaying AIDS progression and reducing low-grade chronic inflammation in HIV infection.

Keywords : Interleukin IL-18 (IL-18), 18 binding protein (IL-18BP), HIV infection, platelets, intestinal permeability.

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List of abbreviations

ADP	Adenosine diphosphate
AIDS	Acquired immune deficiency syndrome
AIM2	Absent in melanoma 2
AJs	Adherens junctions
ART	Antiretroviral therapy
ATP	Adenosine triphosphate
BFA	Brefeldin A
CA	Capsid
CAD	Coronary artery disease
cART	Combination antiretroviral therapy
CD40L	CD40 ligand
CLEC-2	C-type lectin-like receptor 2
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTL	Cytotoxic T lymphocytes
CXCL5	C-X-C motif ligand 5
CXCR	CXC chemokine receptor
DCs	Dendritic cells
DC-SIGN	DC-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
dsRNA	Double-stranded RNA
DSS	Dextran sulfate sodium
EC	Elite controllers
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay

FasL	Fas ligand
FDA	Food and Drug Administration
FOXO3a	Forkhead box transcription factor O class 3a
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
Gp	Glycoprotein
GVHD	Graft-versus-host disease
HAD	HIV-associated dementia complex
HALS	HIV-associated lipodystrophy syndrome
HAART	Highly active antiretroviral therapy
HCE	Human corneal epithelial
HEK 293T	Human embryonic kidney cells 293 expressing the SV40 T-antigen
HIV	Human immunodeficiency virus
HIV-1	HIV type- 1
HIV-2	HIV type- 2
HTLV-III	Human T lymphotropic virus type III
HLA	Human leukocyte antigen
HVL	High viral load
IBD	Inflammatory bowel disease
Ic	Intracellular
IEC	Intestinal epithelial cells
Ig	Immunoglobulin
IGIF	IFN-γ-inducing factor
IFI16	Interferon-γ-inducible protein 16
IFN-γ	Interferon gamma
IL	Interleukin
IL1F11	11^{th} member of IL-1 family

IL-1Ra	IL-1 receptor antagonist
IL-1RAcP	IL-1 receptor accessory protein
IL-18	Interleukin IL-18
IL-18BP	Interleukin-18 Binding Protein
IL-18R	IL-18 receptor
IL-36Ra	IL-36 receptor antagonist
IRAKs	IL-1R–associated kinases
JAM	Junctional adhesion molecule
KSHV	Kaposi's sarcoma-associated herpes virus
LPS	Lipopolysacharide
LTNP	Long-term nonprogressors
LTR	Long-terminal repeat
MA	Matrix
mIL-18	Mature IL-18
MHC	Major Histocompatibility complex
MLC	Myosin light chain
MLCK	Myosin light-chain Kinase
mRNA	Messenger RNA
MyD88	Myeloid differentiation factor 88
NC	Nucleocapsid
Nef	Negative factor
NF-κB	Nuclear factor kappa B
NK	Natural killer
NLRP3	Nod-like receptor 3
NOD	Nucleotide oligomerization domain
NLRs	NOD -like receptors

PBMCs	Peripheral blood mononuclear cells
PDGF	Platelet-derived growth factor
PF4	Platelet factor 4
PI	Protease inhibitors
pIL-18	Precursor IL-18
p-MLC	Phosphorylated myosin light-chain
p-STAT5	Phosphorylated signal transducer and activator of transcription 5
PR3	Proteinase 3
Pre-mRNA	Precursor mRNA
PUMA	p53 upregulated modulator of apoptosis
RA	Rheumatoid arthritis
RANTES	Regulated upon activation, normal T-cell expressed, and presumably secreted
RIG-I	Retinoic acid-inducible gene-I
RLRs	RIG-I like receptors
ROCK	Rho-associated protein kinase
RP	Rapid progressors
siRNA	Small interfering RNA
SIVs	Simian immunodeficiency viruses
SLE	Systemic lupus erythematosus
SNPs	Single nucleotide polymorphisms
SU	Surface unit
TER	Transepithelial resistance
TEER	Transepithelial electrical resistance
TGF-β	Transforming growth factor β
Th 17	T helper type 17

TJs	Tight junctions
TLRs	Toll-like receptors
TLR9	TLR subtype 9
TM	Transmembrane
TNBS	2, 4, 6-Trinitrobenzene sulphonic acid
TNF-α	Tumor necrosis factor alpha
TRAF6	TNF receptor-associated factor 6
VCAM-1	Vascular cell adhesion molecule 1
Vif	Virus infectivity factor
Vpu	Virus protein u
ZO	Zonula occludens

"We all have an unsuspected reserve of strength inside
that emerges when life puts us to the test."
Isabel Allende

"Human beings are not born once and for all on the day their mothers give birth to them,
but ... life obliges them over and over again to give birth to themselves."
Gabriel García Márquez

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Chapter 1:

Introduction

1.1. Human Immunodeficiency Virus

1.1.1 History

Human immunodeficiency virus (HIV) infection and acquired immune deficiency syndrome (AIDS), which are extensively investigated nowadays, were considered a great myth in the early 1980s. In 1981, the U.S. Center for Disease Control and Prevention identified AIDS as a new disease [1]. This came after the discovery of four homosexual men from Los Angeles suffering from complete immune system shutdown, with multiple unexplained viral and bacterial infections associated with Kaposi malignant tumors. At the time, such symptoms were incomprehensible, especially Kaposi's sarcoma, which is a rare malignancy and more often associated with the elderly.

In May 1983, a French research group isolated a new retrovirus from lymphoid tissue obtained from patients with AIDS and named it lymphadenopathy-associated virus (LAV) [2]. Later, in May 1984, a U.S.-based research group confirmed the discovery of this virus believed to be the causative factor in AIDS and labeled it human T lymphotropic virus type III (HTLV-III) [3]. HTLV-III is now known as HIV type 1 (HIV-1) and spreads by sexual, parental, and blood contamination routes. Some years later, a new virus was isolated from patients with AIDS in West Africa and named HIV type 2 (HIV-2). Interestingly, HIV-2 is more closely related than HIV-1 to simian immunodeficiency viruses (SIVs) observed in macaques [4]. SIVs that are relatively closer to HIV-1 and HIV-2 have been detected as nonpathological viruses in chimpanzees and mangabeys. This finding supports the fact that HIV originated as a zoonotic infection in the first patient from Africa [5], and suggests the fact that SIV crossed the species barrier resulting in HIV-1 and HIV-2 in humans.

Soon after HIV/AIDS identification, AIDS became endemic in the United States and many countries worldwide, and was considered as the primary cause of death of people aged between 25 and 44 years. By 1989, the infection had spread in 145 countries and infection levels reached 400,000 cases. Approval of the first commercially available enzyme-linked immunosorbent assay (ELISA) by the U.S. Food and Drug Administration (FDA) in 1985

enabled the detection of new infections. The FDA approved the first HIV protease inhibitor in 1995. This was followed by the discovery of the first highly active antiretroviral therapy (HARRT) in 1997 [6]. HARRT was considered successful treatment for HIV infection following a 40% decrease in AIDS-related deaths in the U.S. during the first year of its introduction to the market.

1.1.2 Pathogenesis of HIV

Although several decades have passed since the discovery of HIV, AIDS and HIV infection are still considered a strong threat to humans. The pathogenesis of HIV infection starts with a variable asymptomatic period before the onset of AIDS symptoms. This period is characterized by different pathogenic processes, such as severe depletion of CD4+ T cells, viral load increase, immune system activation, and intestinal barrier damage. As presented in **Figure 1**, the pathogenesis of HIV infection can by classified into three main phases: (1) acute primary infection, (2) chronic asymptomatic phase, and (3) AIDS.

1.1.2.1 Acute primary infection

The acute primary infection stage includes virus transmission and initial immune responses. HIV successfully infects CD4+ T cells and dendritic cells (DCs) present in mucosal membranes [7]. DCs are specialized antigen-presenting cells responsible for presenting the virus to naïve T cells leading to their activation. Infected DCs and macrophages play an important role in spread of infection by migrating to draining lymph nodes, especially intestinal lymph nodes [8]. Antigen-presenting cells (APC) also indirectly spread the infection by activating CD4+ cells and making them more susceptible to HIV infection. This APC response is preceded by substantial production of proinflammatory cytokines, such as interferon gamma (IFN-γ), interleukin (IL)-6 (IL-6), IL-10, IL-12, and IL-18 from DCs, macrophages, and natural killer (NK) cells [9, 10]. Furthermore, activated CD4+ cells become more susceptible to HIV infection. The production of this storm of cytokines enhances the ability of the virus to infect more CD4+ T cells. Since infected CD4+ T cells are the target of cytotoxic T lymphocytes (CTL) [11], acute primary infection is characterized by an intense decrease in CD4+ T cell count

in the gut mucosa [12]. When infected in vitro, CD4+ T cells mainly die by pyroptosis, a process that involves cellular swelling, cell membrane rupture, and release of intracellular contents in to the extracellular environment [13]. Unlike apoptosis, pyroptosis is a form of cell death associated with production of inflammatory cytokines, such as IL-1b and IL-18 [14]. During the infection process, incomplete reverse transcription products resulting from abortive HIV infection are sensed by the cytosolic DNA sensor, the interferon-γ-inducible protein 16 (IFI16), which leads to inflammasome assembly and activation of caspase-1 [15]. Caspase-1 activation is known to cause cell death via pyroptosis [16].

1.1.2.2 Chronic asymptomatic phase

Depletion of CD4+ T cells due to cytotoxic T lymphocytes and apoptosis continues during the chronic asymptomatic period of HIV infection. Meanwhile, production of new CD4+ T cells in the thymus is disrupted. HIV infection has been shown to strongly affect thymus function and decrease the production of new CD4+ T cells [17]. Furthermore, the immune system fails to clear the virus and viral proteins, which maintains the production of proinflammatory cytokines leading to chronic inflammation. This results into defective intestinal barriers and translocation of bacterial toxins into the circulation, which directly activate DCs and macrophages to produce more proinflammatory cytokines [18]. Continued viral production, together with high viral load and chronic inflammation, dramatically depletes CD4+ T cell count and accelerates AIDS symptoms.

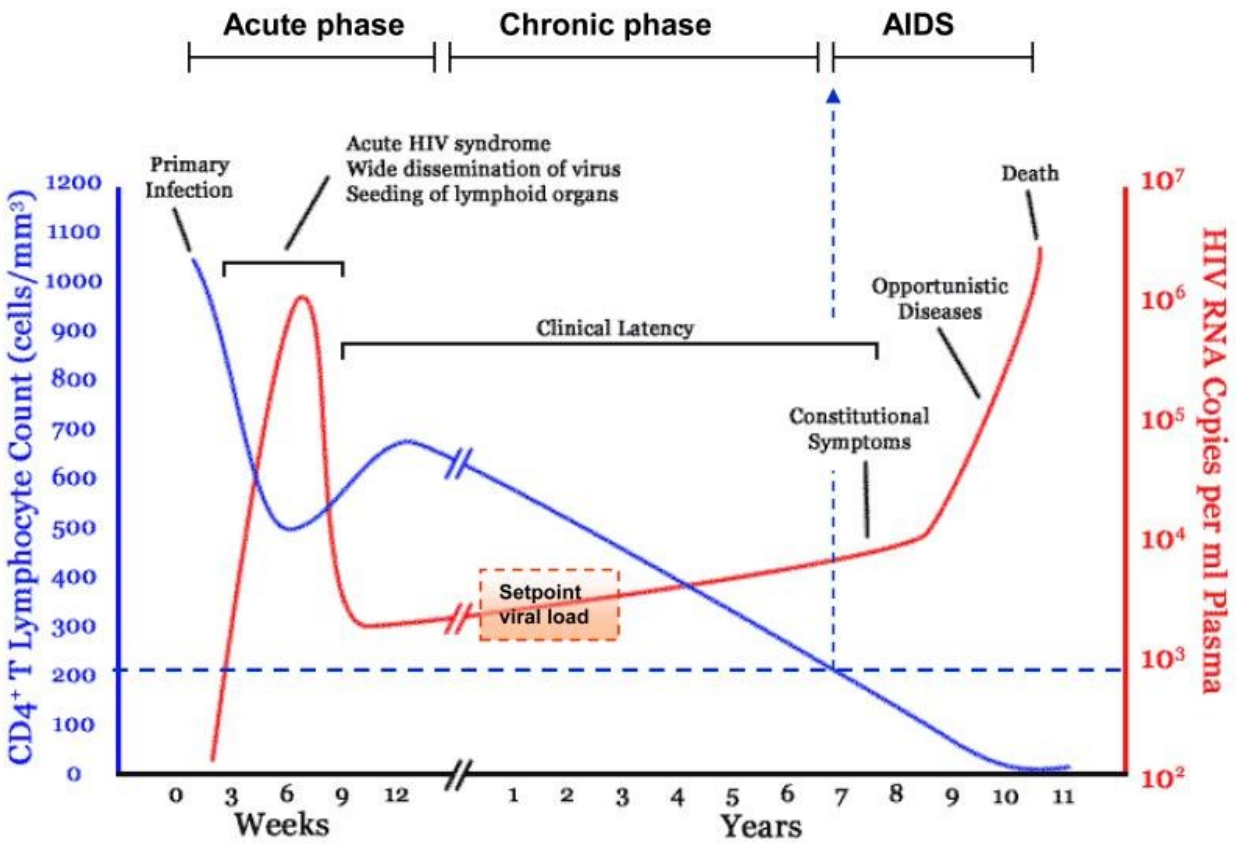

Figure 1. A typical natural time course of HIV infection from an acute infection until the onset of AIDS

The Figure shows the typical time course of HIV infection comprising three phases: acute infection phase, chronic phase, and AIDS. The acute infection phase typically lasts for weeks and involves a sharp reduction in CD4+ T cell count and increase in viral load. The chronic phase is typically asymptomatic and spans several years. Continued viral production and reduced function of the immune system give rise to the symptoms observed during the final AIDS phase.

1.1.3 Structure of HIV

HIV is a lentivirus from the Retroviridae family [20]. HIV-1 and HIV-2 are the two types of viruses that cause AIDS. HIV-1 is distributed worldwide and it is the more virulent type, while HIV-2 is more localized to western and central Africa. As presented in **Figure 2**, HIV contains two identical copies of single-stranded RNA. Both types of virus encode nine open-reading frames responsible for protein production. The three main genes containing the viral structural proteins necessary to build a new virus are *gag*, *pol*, and *env* [21]. The *env* gene encodes glycoprotein (gp)-160, which is cleaved by the cellular enzyme furin to gp120 and gp41 proteins to form the viral envelope. The *gag* gene is cleaved into structural protein products (p24, p7, and p6) to form the viral core. The pol (DNA polymerase) gene is necessary to synthesize viral DNA and integrate it with host DNA to enhance viral reproduction. In addition to *gag*, *pol*, and *env*, HIV has two essential regulatory genes and four important accessory genes. The two essential regulatory genes regulate the expression of virion proteins (*rev*) and HIV trans-activator (*tat*) [22]. Rev protein is responsible for exporting intron-containing HIV-1 RNA from the cytosol to the nucleus during viral replication [23]. Tat protein plays an essential role in regulating the transcription of the full length of the RNA genome. In the absence of *tat*, RNA polymerase II fails to synthesize full-length viral transcripts [24]. The other four regulatory proteins are virus infectivity factor (Vif), viral protein u (Vpu), negative factor (Nef), and viral protein R (Vpr). HIV-1 *vif* encodes the highly conserved 23-kDa Vif protein, which is generated at a late stage of the virus life cycle and confers HIV infectivity [25]. Vif protein binds and degrades APOBEC-3G, an RNA editing enzyme, via proteasomal pathway. This way, Vif prevents the viral RNA from the hypermutating effects of ABOBEC-3G and enhance viral replication [26] Vpu is specific to HIV-1 and involved in CD4+ molecule degradation and deactivation of the nuclear factor kappa B (NF-κB) pathway [27]. Vpu is also capable of inducing pores in the cell membrane to ensure successful release of virus particles (virions) from the infected cells [28]. In the absence of Vpu, newly produced HIV particles remain attached to the cell surface and are not released from the interferon-inducible cellular restriction factor, BST2 or tetherin [29]. HIV-1 overcomes this BST-2-induced host protective mechanism by degrading tetherin via ubiquitination [30]. In contrast, Nef is involved in many functions during virus replication. It protects infected cells from CTL-induced lysis by interfering with the

expression of major histocompatibility complex (MHC) class I antigens [31]. It also causes the release of tumor necrosis factor alpha (TNF-α) from peripheral blood mononuclear cells (PBMCs) and provides microenvironment support for viral replication [32]. Nef activates T cells [33], and reduces the repertoire of antibodies produced by B cells by inhibiting the switch from immunoglobulin (Ig)-M (IgM) to IgA and IgG [34]. Vpr is a 14-kDa multifunctional protein that increases permissiveness and viral production [35]. It also plays an important role in promoting provirus entry into the nuclei of nondividing cells [36]. Moreover, this protein may participate in HIV-1 NK cell dysfunction by up-regulating NKG2D ligands and promote NK cell mediating killing of the virus-infected cells [37]. Also, Vpr has a substantial role in activation of long-terminal repeats (LTR) transcription [35]. The HIV transcription promoter is encoded by two LTRs: LTR3 and LTR5.

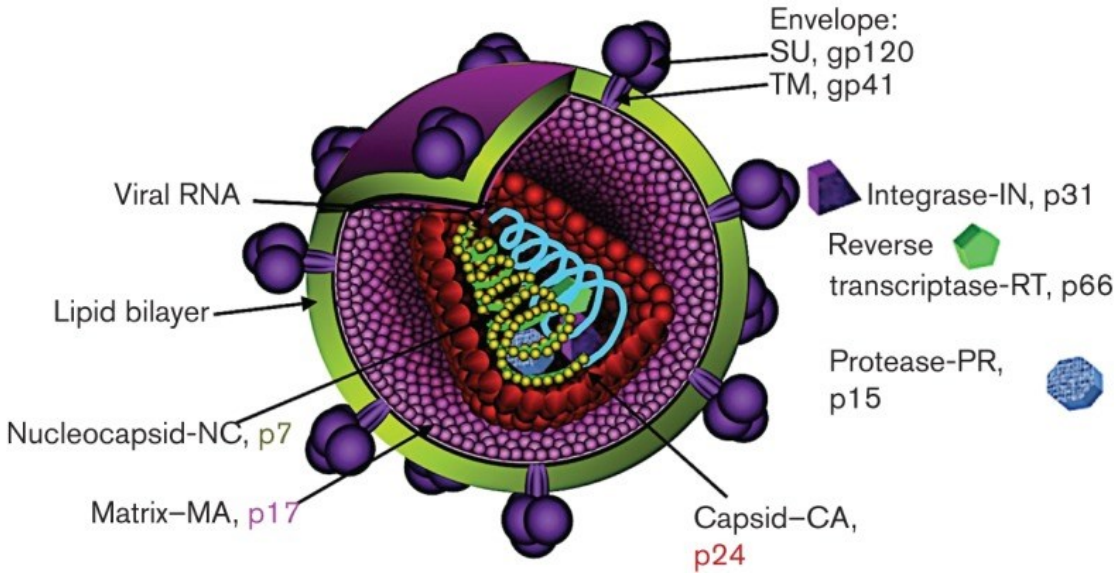

Figure 2: HIV-1 virion structure.

HIV virion main structures, envelope, capsid, matrix, nucleocapasid, lipid bilayer and viral RNA with detailing the localization of viral proteins. Abbreviations: SU, surface unit; gp120, glycoprotein 120; TM, transmembrane; gp41, glycoprotein 41.

1.2. Intestinal Permeability

1.2.1 Structure of the Epithelial Barrier

The intestinal tract comprises polarized monolayer epithelial cells, which are attached to each other by a complex structure known as the epithelial barrier. The intestinal epithelial barrier is a protective physiological barrier that regulates the transportation of water and nutritional particles. It also prevents bacteria, bacterial toxins, degraded food particles, and digestive enzymes from entering the circulation [39]. Different substances can be transported across intestinal barriers by paracellular or transcellular transport. Paracellular intestinal transport refers to small molecules that can pass between epithelial cells through the intercellular complex [40]. In contrast, transcellular intestinal permeability describes the process whereby substances pass through individual cells. As presented in **Figure 3**, the intercellular complex is composed of three structures: tight junctions (TJs), adherens junctions (AJs) and desmosomes, [41]. TJs and AJs are found on the apical side and play an essential role in the formation and maintenance of the integrity of the barrier. Disruption of these junctions induces intestinal leakage and increases antigenic and lipopolysaccharide (LPS) presentation to the immune system.

TJ structures are predominantly constructed from two components: transmembrane proteins, including occludin, junctional adhesion molecule A (JAM-A), and members of the claudin family. Cytoplasmic TJs proteins include zonula occludens types 1–3 (ZO-1, ZO-2, and ZO-3), cingulin, and afadin [42]. Both transmembrane proteins and cytoplasmic proteins are directly linked with underlying cytoskeletal actin filaments [43]. AJs, which are predominantly composed of β-catenin and p120 catenin, are in direct contact with E-cadherin and its actin cytoskeleton intracellular domain. Furthermore, the apical side of intestinal epithelial cells contains an abundance of actin filaments (F-actin) and nonmuscle myosin II that reorganize the actin cytoskeleton. F-actin is found as a circumferential belt at the level of the AJs and as a dense meshwork at the level of the TJs. Depolymerization of F-actin plays a principle role in epithelial barrier integration and junction function [44].

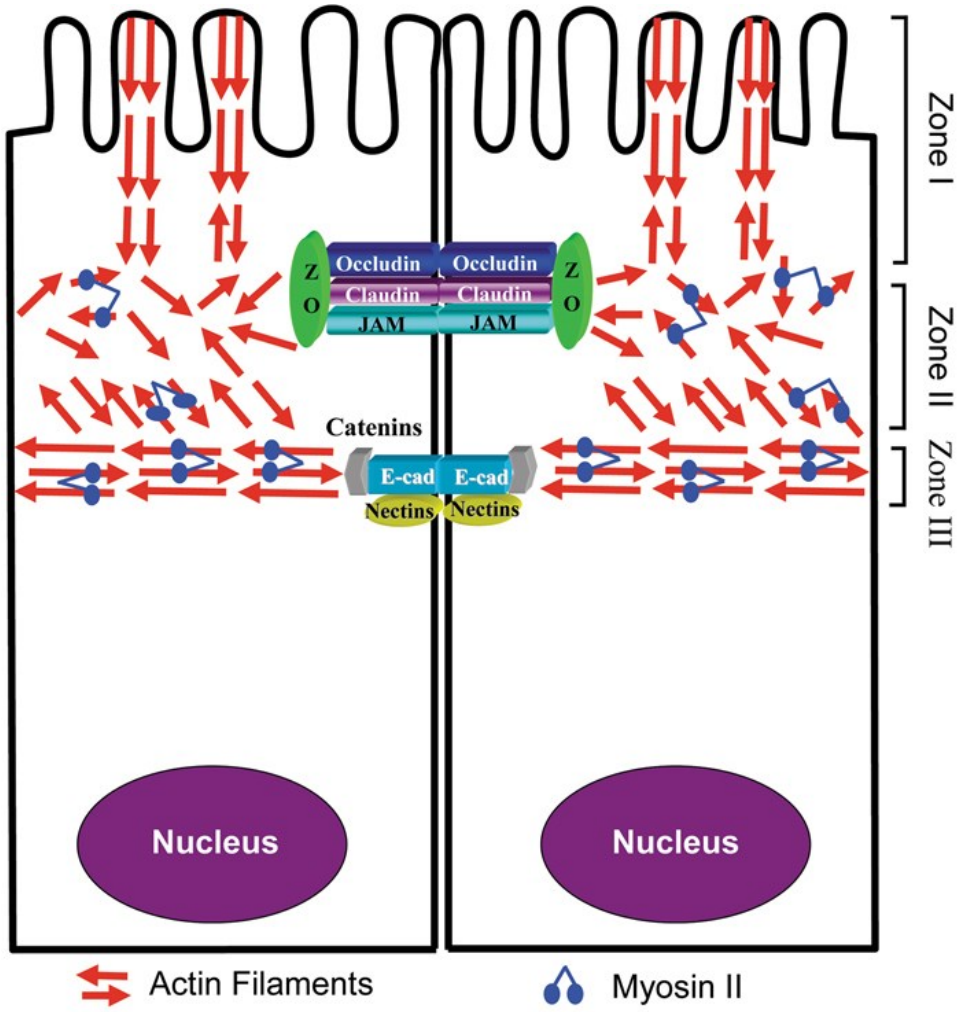

Figure 3: Structure of epithelial barrier

The **Figure** shows the distribution of Actin Filaments (F-Actin) and non-muscle myosin II (NM II) in intestinal cells. It demonstrates also the location of tight junctions (TJ) proteins such as: zonula occludens (ZO), claudin, occludin and junctional adhesion molecule (JAM) and adherens junctions (AJs) proteins such as catenins, E-cadherin, and nectins.

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1.2.2 HIV Infection and Intestinal Permeability

Histological abnormalities of the intestinal mucosa were one of earliest observations made in 1984 in patients with AIDS [45]. Later on, the medical term *HIV enteropathy* was established to describe HIV-associated inflammation, diarrhea, malabsorption, and increased intestinal permeability. Interestingly, HIV-associated morphological changes in the GI epithelial layers, such as crypt hyperplasia and villous atrophy, occur without detection of further pathogens [46]. Intestinal permeability increases five-fold in HIV patients compared with healthy individuals [47]. This increase in permeability correlates with the significant increase in plasma LPS in patients with chronic HIV. In addition, progression of HIV toward AIDS correlates with circulating LPS levels. Interestingly, HAART decreases the level of plasma LPS in treated patients [18]. In support of this observation, LTNP and EC patients have less viral replication in the GI tract, and this reflects on LPS plasma levels [18, 48].

The mechanisms by which HIV leads to mucosal barrier dysfunction are yet to be established. Several researchers have demonstrated the direct effect of the virus itself, and of viral proteins, in breaching the intestinal barrier. Incubating the T84 intestinal cell line with different strains of HIV virus in vitro for 24 hours generated several interesting findings [49]. HIV downregulates production of occludin, ZO-1, and claudins 1–5, and disrupt TJs. Cells exposed to HIV exhibit decreased transepithelial resistance (TER). TER and transepithelial electrical resistance (TEER) are two related techniques to measure permeability but differ in methodology. Furthermore, bacterial and viral translocation across intestinal cells occurs after cells are treated with HIV [49]. Interestingly, HIV-related proteins play a key role in affecting epithelial barriers in different organs. HIV-1 Tat protein induces oxidative stress and causes alveolar epithelial dysfunction in animal models [50]. In the intestinal epithelial cell line Caco2, Tat protein induces caspase-3 activation and causes apoptotic cell death [51]. Tat protein inhibits cells proliferation and affects intestinal cells integrity by decreasing TEER [52]. Moreover, HIV-1 gp120 plays an important role in increasing intestinal permeability. gp120 decreases TER

and stimulates microtubule depolymerization in the intestinal cell line HT29 [53]. TER, which decreased in the T84 cell line after incubation with HIV, increased following treatment with a gp120 antibody [49]. On the other hand, HIV infection is associated with upregulation of many proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6. These cytokines have a principal effect on intestinal permeability, which will be addressed in a later section of this thesis.

1.2.3 HIV Infection and the GI Immune System

It is widely believed that the intestinal immune system is central to the development of HIV pathogenesis. Most of the essential viral infection events, such as transmission, viral replication, and CD4+ T cell destruction are localized to intestinal tissue. Statistics indicate that 90% of worldwide infections are transmitted by genital mucosa and intestinal tissue [54]. This is because the intestine is the largest immunological organ in the human body, and contains almost 40% of lymphocytes, the majority of the T cell population, and lymphoid follicles forming Peyer's patches [55, 56]. In addition, CD3+ T cells compose 90% of intraepithelial lymphocytes [57].

1.2.4 Cytokines and Regulation of Intestinal Permeability

Different inflammatory conditions, such as Crohn disease, ulcerative colitis, and irritable bowel syndrome, or infections due to bacteria, such as *Clostridium difficile* and *Vibrio cholerae* disrupt the intestinal barrier and produce a "leaky" intestine. Increased intestinal permeability enhances antigenic penetration into the lamina propria. As shown in **Figure 4**, antigen-presenting cells, such as DCs, macrophages, and T helper cells, respond to this invasion of antigens and produce proinflammatory cytokines and results in disrupted intestinal barrier. The role of key proinflammatory cytokines in increasing intestinal permeability will be discussed in further detail.

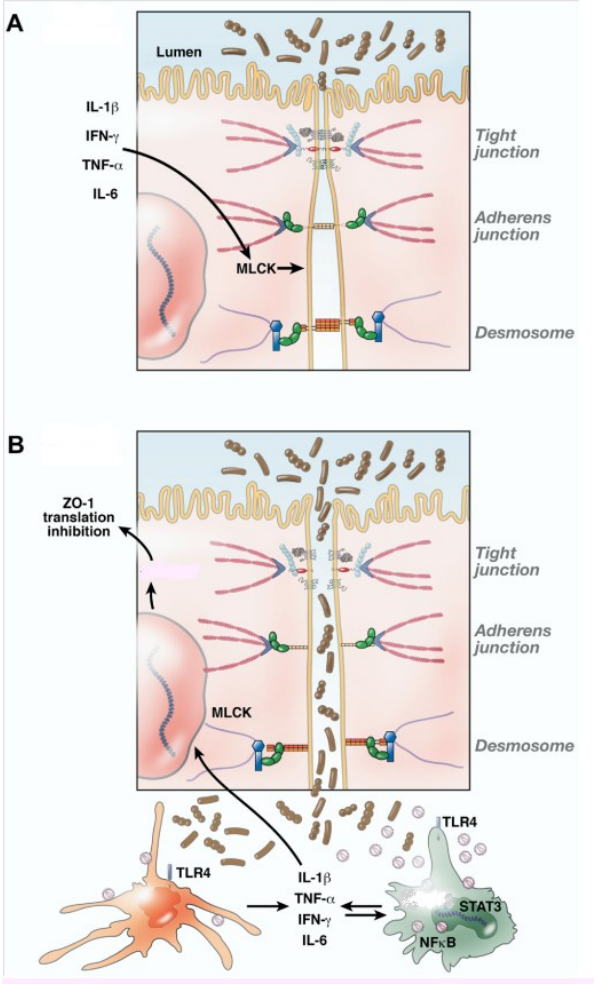

Figure 4: Possible molecular mechanisms implicated in intestinal epithelial barrier disturbance

(A) Normal structure of intestinal epithelial barrier with normal junctional complexes including tight junctions, adherens junctions and desmosomes. The Figure also shows the role of proinflammatory cytokines such as IL-1β, TNF-α, INF-γ and IL-6 in initiating myosin light-chain kinase (MLCK) expression and activation. (B) Disrupted epithelial barrier results in activation of macrophages and DCs through TLRs, and increase in proinflammatory cytokine production by activating STAT3 and NFκB pathways. This leads to further dysfunction of the barrier and increased permeability through *Zonula occludens-1 (ZO-1) translocation*.

1.2.4.1 IFN-γ

The potential for cytokines to increase intestinal permeability was first reported in 1998. *Madara et al.* showed a dose- and time-dependent increase in the TJ permeability of T89 intestinal cells in the presence of IFN-γ [59]. IFN-γ reduces TEER in T84 monolayer cells, which is prevented in the presence of anti–IFN-γ antibodies that block IFN-γ receptors [60]. *Watson et al.* proposed that TJs have two different types of pores; one responsible for large size molecules and the other for smaller molecules. The authors also demonstrated the ability of IFN-γ to permit large-sized molecules, but not small-sized molecules, to cross TJs [61]. IFN-γ affects intestinal junctions proteins, occludin, claudin-1, and JAM-A by different mechanisms. IFN-γ increases endosomal uptake of occludin through macropinocytosis and disrupts TJs [62]. This property requires phosphorylation of myosin light chain (MLC). Furthermore, IFN-γ–induced reduction in TEER is reversed by inhibiting Rho kinase activation and by using phosphatidylinositol 3'-kinase (PI3-kinase) inhibitors [63, 64].

1.2.4.2 IL-1β

IL-1β is a proinflammatory cytokine belonging to the IL-1 family. IL-1β is produced by many immune cells, such as eosinophils, macrophages, T cells, and DCs [65-67]. Treatment of Caco2 cells with IL-1β induces the expression of other inflammatory cytokines, e.g., TNF-α and IL-8 [68]. A significant and dose-dependent decrease in TEER (80% reduction following three days of treatment) is reported with IL-1β on the basolateral but not on the apical side [68]. IL-1β causes a time-dependent disruption in TJs by decreasing occludin and redistributing claudin-1, and this effect is prevented in the presence of NF-κB inhibitors [69]. The increase in Caco2 permeability induced by IL-1β is associated with an increase in MLCK protein level [70]. However, IL-1β affects the distribution of TJ and AJ proteins in corneal pigment epithelial cells, and decreases TEER in simian virus 40-transformed human corneal epithelial (HCE) cells [71]. A similar effect was observed in pulmonary epithelial cells [72].

1.2.4.3 TNF-α

TNF-α is mainly produced by activated macrophages and T lymphocytes during inflammation. It also plays an essential role in inducing necrosis in tumor cells [73]. Interestingly, TNF-α is undetectable in healthy people, while it is detectable in tissues and serum

during inflammation or infection. The ability of TNF-α to increase epithelial permeability was reported in different cells types. TNF-α dose-dependently decreased TER in retinal epithelial cells [74]. Similarly, TNF-α is associated with increased intestinal permeability in Caco2 and T84 cells [75, 76]. However, TNF-α more aggressively disrupted epithelial TJs in the HT29 cell line. Treatment of HT29 with 100-ng/mL TNF-α induced an 81% decrease in TER after 24 hours. This response was reversed in the presence of tyrosine kinase and protein kinase A inhibitors [77]. Several researchers have demonstrated the principal role of MLCK protein to increase intestinal permeability by cytokines, such as IFN-γ and IL-1β. For example, *Ma et al.* demonstrated correlation between increased Caco2 TJ permeability in response to TNF-α and elevation in MLCK [78]. Similarly, blocking NF-κB inhibits elevation in MLCK gene expression and TJ disruption [78]. In contrast to IFN-γ and IL-1β, TNF-α induces apoptosis by activating the caspase cascade. Treating HT29 cells with TNF-α enhances apoptosis two fold compared with untreated cells. This could promote greater intestinal permeability by increasing the gaps between adjacent intestinal epithelial cells [79].

1.2.4.4 Transforming growth factor-β

Transforming growth factor β (TGF-β) is a multifunctional cytokine produced by many cells, including macrophages and intestinal epithelial cells. It controls cell proliferation and differentiation. Unlike many other cytokines, TGF-β enhances the function of the intestinal barrier by increasing expression of claudin-1 [80]. TGF-β augments basal resistance, as measured by TEER, of the T84 colonic epithelial cell line. It also shows strong ability to reverse the decrease in TEER induced by IFN-γ [81]. Furthermore, it inhibits the disturbance in TJs caused by *Escherichia coli* O157:H7 [80]. However, this protective effect toward epithelial barriers is selective for intestinal tissue. *Woo et al.* reported that TGF-β disrupts breast epithelial cells in an animal model [82]. In testicular Sertoli cells, TGF-β increases expression of occludin and other TJ proteins, and disrupts the epithelial barrier [83].

1.3. Platelets as Immune and Inflammatory Cells

1.3.1 Platelets as Immune Cells

Platelets are the second most numerous cells in the human body and are only surpassed in number by red blood cells. In humans, there are approximately 200,000 platelets per μL of blood [84]. They are very small anuclear circulating cells, with an approximate diameter of 1–2 μm and lifespan between 8 and 10 days. They are shed in the blood stream from megakaryocytes, their progenitor cells. In contrast to platelets, megakaryocytes predominantly reside in bone marrow and are very large polyploid nuclei cells. Platelets contain approximately 60 different granules, which are classified under four types: (1) α-granules, (2) dense granules, (3) lysosomal granules, and (4) T granules [85]. The largest number and variety of platelet proteins are stored in α-granules, of which 50–60 are present per platelet. Proteomic analysis demonstrated that α-granules contain 284 proteins [86]. It is interesting to note that α-granules are the largest of platelets granules (200–400 nm in diameter) and secrete many important immunological proteins, such as like platelet factor 4, CXC chemokine receptor (CXCR) types 1–4, platelet-derived growth factor, TGF-β, and RANTES (Regulated upon activation, normal T-cell expressed, and presumably secreted) [87]. Dense granules are the second most common type of platelet granules. These are smaller (~150 nm in diameter) and less in number (3–8 per platelet) than α-granules. Dense granules contain molecules, such as adenosine triphosphate (ATP), adenosine diphosphate (ADP), serotonin, and glutamate [88, 89], and play a key role in inflammation by interacting with immune cells and amplifying inflammatory responses. Lysosomal granules are sparse; however, these granules contain degradative enzymes, such as aryl sulfatase, that play a role in wound healing and repair [90]. T granules were most recently discovered and play a role in TLR subtype 9 (TLR9) organization and function [91].

Platelets play an essential role as cellular mediator of thrombosis and hemostasis. In addition, platelets are considered as the most numerous immune cells. By producing serotonin, platelets participate in differentiation of monocytes to DCs and activated naïve T cells [92, 93]. Platelets are a major source of CD40 ligand (CD40L), which has been shown to increase T cell immunity to viral infection and induce DC maturation [94]. A mouse model showed that Tat

causes shedding of CD40L from platelets [95]. Platelets also release RANTES, which recruits leukocytes to inflammatory sites and plays a role in the differentiation of NK cells [96]. Furthermore, platelets express different members of the TLR family, such as TLR1, TLR4, TLR6, TLR8, and TLR9 [97]. LPS interacts with TLR4 to activate platelets that induce neutrophils to form neutrophil extracellular traps to restrict bacterial invasion [98]. Platelets are also considered as a source of several proinflammatory cytokines, such as IL-6, IL-8, and IL-1β [99].

1.3.2 Platelets and IL-1β Synthesis

It was thought for some time that platelets solely released premade proteins upon activation. The ability of activated (but not inactivated) platelets to express IL-1 was demonstrated in the late 1980s [100]. Surprisingly, *Denis et al.* demonstrated the presence of precursor mRNA (pre-mRNA) in platelets and proplatelet extensions of megakaryocytes. Activation of platelets by thrombin has been shown to splice IL-1β pre-mRNA to mature mRNA and synthesize IL-1β protein [101]. Interestingly, platelet synthesis of IL-1β is associated with clot formation and maturation. Use of monoclonal antibodies to block β3 integrins markedly decreases IL-1β synthesis and prevents fibrin mesh retraction [102].

1.3.3 Platelets and HIV Infection

Thrombocytopenia is one of the most common complications of HIV infection and occurs in more than 25% of AIDS patients [103]. It is also one of the first HIV symptoms exhibited in the acute phase of the infection [104]. The occurrence of thrombocytopenia following the viral infection is multifactorial in origin; it may be the result of the devastation caused by platelet-associated IgG, hypersplenism, inhibited production of thrombopoietin, and the harmful effect of the virus on platelets [105]. HIV infection initiates platelet activation and there appears to be a correlation between disease severity and degree of platelet activation [106]. Furthermore, changes in the morphology of platelets has been observed in HIV/AIDS patients [107]. Although HAART administration remarkably increases platelet count in patients [108], the role of platelets to enhance or clear the infection is still very controversial. It is not yet clear

whether platelets participate in spreading infection by internalizing HIV, or whether platelets aid immune cells to recognize and destroy the virus. *Zucker-Franklin et al*. reported that platelets and megakaryocytes internalize HIV [109]. It has been suggested that virus particles are endocytosed by platelets, enclosed within platelets, or come into contact with platelet α-granules. Two mechanisms for HIV entry into platelets have been proposed: (1) the virus fuses with CD4+ coreceptors, and (2) the virus is endocytosed by DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN). Platelets do not express CD4, the primary HIV receptor; however, they do express CXCR1, -2, and -4 coreceptors [110]. HIV has been shown to bind to platelets via DC-SIGN [111, 112], which is expressed by DCs, DC-derived monocytes, and lymphoid tissue. DC-SIGN–expressing cells internalize HIV-1 and retain viral infectivity for days before infecting T cells [113]. Approximately 15% of platelets express DC-SIGN. This finding supports the notion that HIV endocytosis occurs via functional DC-SIGN [105]. In addition to DC-SIGN, HIV-1 can be captured by C-type lectin-like receptor 2 (CLEC-2) that is also expressed by platelets. CLEC-2 has been identified as a platelet activating receptor for the snake venom rhodocytin [114]. In contrast to DC-SIGN, CLEC-2 facilitates HIV-1 capture by platelets independent of viral envelop protein. CLEC-2 inhibitors clearly reduce HIV-1 binding to platelets [112]. Furthermore, knock-down of the CLEC-2 ligand, mucin-like membranous glycoprotein podoplanin, in human embryonic kidney 293 contains the SV40 T-antigen (HEK 293T) cells by short hairpin RNA results in a strong decrease in HIV-1 transmission to platelets after incubation with infected 293T cells [115]. More research into the role of platelets in HIV infection is necessary since it is unclear how platelets affect the pathogenesis of HIV.

1.4. Interleukin 18

IL-18 is a multifunctional pro-inflammatory cytokine, which belongs to the IL-1 famaily (IL-1F) of cytokines. The family family members are briefly discussed below.

1.4.1 Interleukin 1 Family

The IL-1 family comprises 11 members classified into three subfamilies: (1) the IL-1 subfamily consisting of IL-1α, IL-1β, IL-1RA, and IL-33, (2) the IL-18 subfamily consisting of IL-18 and IL-37, which have shorter propieces than IL-1 and IL-33, and (3) the IL-36 subfamily containing the shortest propieces, such as IL-36α, IL-36β, IL-36γ, IL-38, and IL-36Ra (reviewed in ref [116, 117]). A pro-piece is the N-terminal part that is removed from the precursor by proteolytic cleavage to generate mature and functional form of a cytokine. Most members of the IL-1 family are proinflammatory cytokines, except IL-37, which is an anti-inflammatory cytokine. This family has four signalling receptors: IL-1R1 and IL-1 receptor accessory protein (IL-1RAcP) for IL-1, ST2 and IL1RAcP for IL-33, IL-18Rα and IL-18Rβ for IL-18, and finally IL-1RAcP and IL-1Rrp2 for IL-36. Also, there are two decoy receptors in the IL-1 family, IL-R2 and IL-18BP [116]. The following paragraphs summarize the immunological functions of IL-1 family members, with particular emphasis on IL-18, which will be described in detail.

The IL-1α precursor is present in many organs, such as kidney, liver, lung, and the GI tract. Furthermore, it is also expressed by several cells, especially monocytes and B lymphocytes [118]. Precursor and processed mature forms of IL-1α are released by endothelial cells undergoing apoptosis [119]. Precursor IL-1α is cleaved by calpain, a calcium-activated cysteine protease, and processed into mature IL-1α [120]. Both forms of IL-1α are functional and considered alarming cytokines because these forms initiate many inflammatory reactions [121]. In contrast to IL-1β, IL-1α is localized in the nucleus and moves to the cytosol in cells undergoing necrosis [122].

Unlike IL-1α, precursor IL-1β is not functionally active, and needs to be cleaved by caspase-1 to produce its active form. Also, caspase-1 needs to be processed by newly assembled inflammasomes to become active [123]. However, processing of mature IL-1β is also induced

by non–caspase-1 mechanisms in caspase-1 knockout mice [124], and non–caspase-1 cleavage of precursor IL-1β is believed to be mediated by proteinase 3 (PR3) in neutrophils [125]. IL-1β is produced by monocytes, DCs, macrophages, NK cells, and B lymphocytes. It serves many immunological functions, especially in T cell activation. In the presence of IL-6 and TGF-β, IL-1β stimulates T helper type 17 (Th17) cell development [126]. IL-1β in combination with IL-23 also initiates the development of Th17 [126]. IL-1β is necessary for NK cells to produce IL-22 and for NKT cells to produce IL-17 [127]. Furthermore, IL-1β plays an important role in chronic inflammatory diseases, such as type 2 diabetes, and cancer angiogenesis and metastasis [128, 129]. In animal models, IL-1β–deficient mice have lower inflammatory responses compared with wild-type mice [130], and develop fewer tumors compared with wild-type or IL-1α-deficient mice [131]. The effects of IL-1 in inducing fever and activating the hypothalamus–pituitary–adrenal axis are well described [117].

IL-33, which is also known also as 11th member of IL-1 family (IL1F11), is cleaved by caspase-1, as with IL-1β and IL-18. However, in contrast to IL-1β and IL-18, precursor IL-33 (30 kDa) is biologically active, whereas its processed forms are less active [132]. Like the precursor form of IL-1β, IL-33 is also processed by PR3 in neutrophils [133]. IL-33 binds to the ST2 receptor and IL-1RAcP coreceptor, and the soluble forms of ST2 and IL-1RAcP negatively regulate IL-33 [134]. IL-33 is predominantly produced by endothelial cells, epithelial cells, and fibroblasts, and can amplify both Th2 and Th1 responses to tumors and viral infection. In the presence of IL-12, IL-33 induces IFN-γ production by CD8+ and NK cells. On the other hand, IL-33–activated DCs support polarization of Th2 cells [135]. IL-33 plays an essential role in allergic lung inflammation since it enhances type 2 inflammation [136]. Interestingly, IL-33 shows impressive cardioprotection [137], and has been shown to reduce atherosclerotic plaque formation [138]. In HIV infection, low levels of IL-33 and high levels of its natural antagonist, ST2, were detected in the sera of HIV/AIDS patients compared with healthy uninfected individuals [139]. Unfortunately, no clear data about the role of IL-33 in HIV progression are available, and this field certainly warrants more attention.

IL-1RA is the natural antagonist of the cytokine IL-1. It binds to IL-1R, but it does not recruit IL-1RAcP, thereby preventing IL-1 biological responses [140]. Four isoforms of IL-1RA

exist. The sIL-1RA isoform, which is secreted by virtually every cell that produces IL-1, except endothelial cells, and three other intracellular (ic) isoforms icIL-1RA1, -2, -3, which are found in a variety of cells, such as activated fibroblast, fibroblast-like COS cell line, and the human liver carcinoma HepG2 cell line [140, 141]. IL-1RA knockout mice are smaller than wild-type mice and intraperitoneal LPS injection appears to be more frequently lethal in the knockout than wild-type mice [142]. In human, elevated IL-1RA is observed in patients with immune diseases, such as chronic rheumatic disease, whereas reductions are observed in pancreatic islets from patients with type 2 diabetes [143, 144].

Among the IL-1 family of cytokines, IL-37, which is also termed IL-1F7, is the cytokine that possesses anti-inflammatory characteristics. It is classified as belonging to the IL-18 subfamily and has five isoforms, with IL-37b being the most effective. Similar to IL-1α and IL-33, IL-37 is translocated to the nucleus after activation, which is induced by caspase-1 cleavage [145]. TGF-β is the most efficient stimulus for IL-37 production [146], and using siRNA to block production of IL-37 induces IL-1β production by two- to three-fold [146]. IL-37 transgenic mice have very low inflammatory responses and are protected against LPS challenge, dehydration, acidosis, and hyperkalemia. Also, these mice have low levels of inflammatory cytokines, such as IL-6 and TNF-α [146]. No data are available concerning IL-37 during HIV infection.

The IL-36 subfamily consists of five cytokines: IL-36α, IL-36β, IL-36γ, IL-38, and IL36Ra. The three IL-36 isoforms α, β, and γ, are also known as IL-F6, IL-F7, and IL-F8 and possess agonist characteristics [147]. IL-36 cytokines are expressed by various cells, such as bronchial epithelial cells, monocytes, macrophages, brain tissue, and keratinocytes [148]. LPS has been shown to induce IL-36γ, but not the α and β isoforms, from THP-1 [148]. However, T lymphocytes express both IL-36α and IL-36β [149]. Like IL-18 and IL-1β, IL-36 needs to be processed to become fully active, however, the enzyme responsible for IL-36 activation is still unknown [147]. Nonprocessed IL-36 is less active than its processed form [147]. IL-36α overexpressing transgenic mice suffer from acanothosis and hyperkeratosis skin lesions [150]. Although rhinovirus infection in human is associated with high expression of IL-36γ from

bronchial epithelial cells [151], no information is available about the role of IL-36 cytokines during viral infections like HIV, hepatitis C, or influenza.

IL-36 receptor antagonist (IL-36Ra) is the natural antagonist for several forms of IL-36 and can inhibit NF-κB activation induced by IL-36 in the jurkat cell line [152]. The main role of IL-36Ra is regulation of skin inflammation. Deficiency of IL-36Ra in IL-36α transgenic mice increases skin lesions [150]. Clinical data shows a positive effect of using anti-TNF in psoriasis patients and this improvement is associated with a decrease in IL-36 agonist and IL-36Ra [153]. A few reports have discussed the role of IL-36 and IL-36Ra in other organs, such as lung and joints [154, 155]. This area of research area remains to be explored.

IL-38, also known as IL-F10, is the 10^{th} member of the IL-36 subfamily. It shares 40% homology with both IL-36Ra and IL-1RA [156]. Precursor IL-38 is not cleaved by caspase-1 as it does not have a caspase-1 cleavage site. IL-38 is expressed in skin epithelial cells and proliferating B cells of the tonsils [157]. Although very little is known about the biological function of IL-38, it was recently reported that IL-38 reduces Th17 responses to heat-inactivated *Candida albicans* [158]. Different ligands and receptors for the IL-1 family are presented in **Figure 5**.

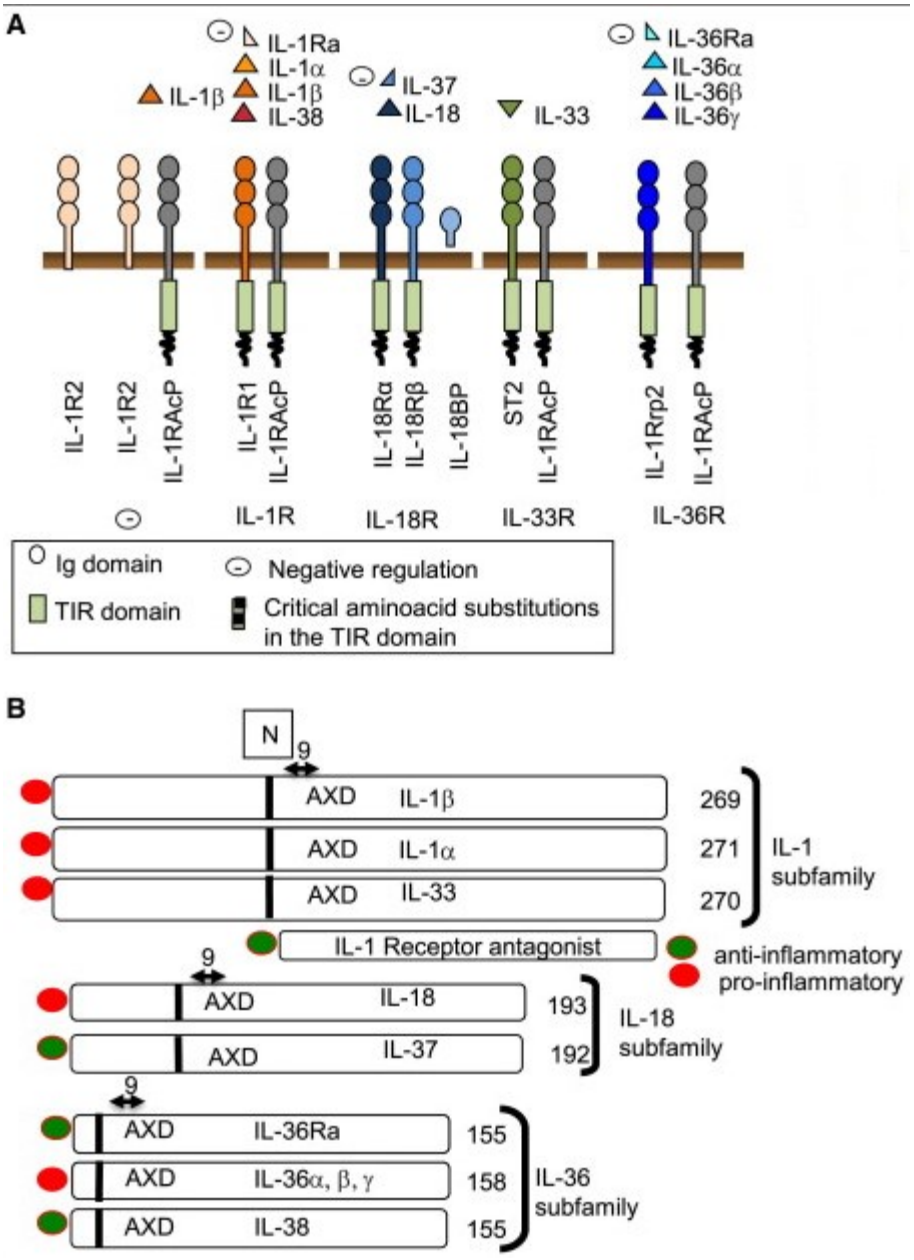

Figure 5: Ligands and Receptors in the IL-1 Family.

(A) A schematic demonstration of ligands and receptors of the IL-1 family. (B) Subfamilies among IL-1 ligands, classified by the length of the N-terminal prodomain. Numbers related to amino acids sequence.

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1.4.2 IL-18 Production and Activation

IL-18 was discovered in 1987 in the serum of mice injected intraperitoneally with heat-killed *Propionibacterium acnes* and challenged with LPS [159]. It was cloned for the first time from the liver of these mice and was termed IFN-γ-inducing factor (IGIF) [160]. IGIF, which is now known as IL-18 and also termed IL-1F4, consists of 192 amino acids for the precursor form and 157 amino acids for the active form [161]. The gene encoding IL-18 gene is ocated on chromosome 11 in humans and chromosome 9 in mouse [162, 163]. IL-18 is a member of the IL-1 family and shares characteristics with some IL-1 family members. Comparable to IL-1β, IL-18 is synthesized in its inactive precursor form and remains intracellularly located. Many cells in the body can produce IL-18, and expression of IL-18 has been detected in macrophages, DCs, monocytes, and Kupffer cells [164, 165]. In addition, intestinal epithelial cells and osteoblasts can produce IL-18 [166, 167]. Activation and processing of the mature form of IL-18 can occur by canonical and noncanonical pathways.

The canonical IL-18 processing pathway involves cleavage by caspase-1. Caspase-1 itself is activated by assembling Nod-like receptor 3 (NLRP3) inflammasomes and other intracytoplasmic sensors like IFI16 and AIM2. The assembled inflammasome consists of pro–caspase-1, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and NLRP3. Inflammasomes involving NLRP have been shown to assemble after threat signals, such as bacterial toxins, environmental pollutants, or viral infections [168, 169]. Necrotic cells that release ATP can also induce inflammasome assembly [170]. After inflammasome assembly, active caspase-1 is released and cleaves the 24-kDa precursor of IL-18 to release 18 kDa mature IL-18 that induces IFN-γ [171].

Unexpectedly, caspase-1–deficient mice reportedly produce IL-18 [172], and this finding was explained by showing different noncanonical production pathways for IL-18. Omoto et al. described the ability of granzyme B to cleave precursor IL-18 and produce biologically active IL-18 in caspase-1–deficient cells [173]. Similar to IL-1β, PR3 induces secretion of active IL-18 in the presence of a caspase-1 inhibitor from human oral epithelial cells

[174]. In addition, merpine-β, a tissue-specific metalloproteinase, can process pro–IL-18 to its active form. Merpine-β knockout mice appear to have low serum levels of IL-18 [175]. Fas/FasL interaction is another noncanonical production pathway for IL-18. Kupffer cells that expressed Fas were obtained from liver cells in mice treated with *Propionibacterium acnes*. Interestingly, these cells produce active IL-18 when cultured with recombinant FasL or cells expressing FasL [172]. A recent study by *Bossaller et al.* described that Fas signaling induces active caspase-8 in macrophages and DCs, which results in the expression of active IL-18 and IL-1β [176]. On the other hand, caspase-3 can cleave both precursor and mature IL-18 to two inactive products in the THP-1 cell line [177].

1.4.3 IL-18 Receptors and Signaling

The IL-18 receptor (IL-18R) is a heterodimer containing IL-18Rα and IL-18Rβ. IL-18 signaling occurs when free active IL-18 binds to the IL-18Rα receptor. Although IL-18 binds to IL-18Rα with low affinity, this binding results in the recruitment of the coreceptor IL-18Rβ and forms a high affinity complex that initiates intracellular signaling [178]. After the formation of the IL-18/IL-18R complex, a signaling cascade is initiated through myeloid differentiation factor 88 (MyD88). Then, phosphorylation of IL-1R–associated kinases (IRAKs) takes place followed by IRAK dissociation from the complex and binding to TNF receptor-associated factor 6 (TRAF6). Finally, TRAF6 activates NF-κB and induces a signal [179]. See [**Figure 6**] for the signalling cascade initiated by IL-18 binding to its receptor. This signaling pathway is supported by observations in animal models. MyD88 knockout mice are not able to develop IL-18–mediated responses [180]. Furthermore, absence of IL-18Rβ prevents IL-18 signaling. For example, the human lung epithelial cell line A549 lacks IL-18Rβ. No IL-18 signal can be induced until IL-12 is present. IL-12 has been shown to induce expression of the missing receptor [181].

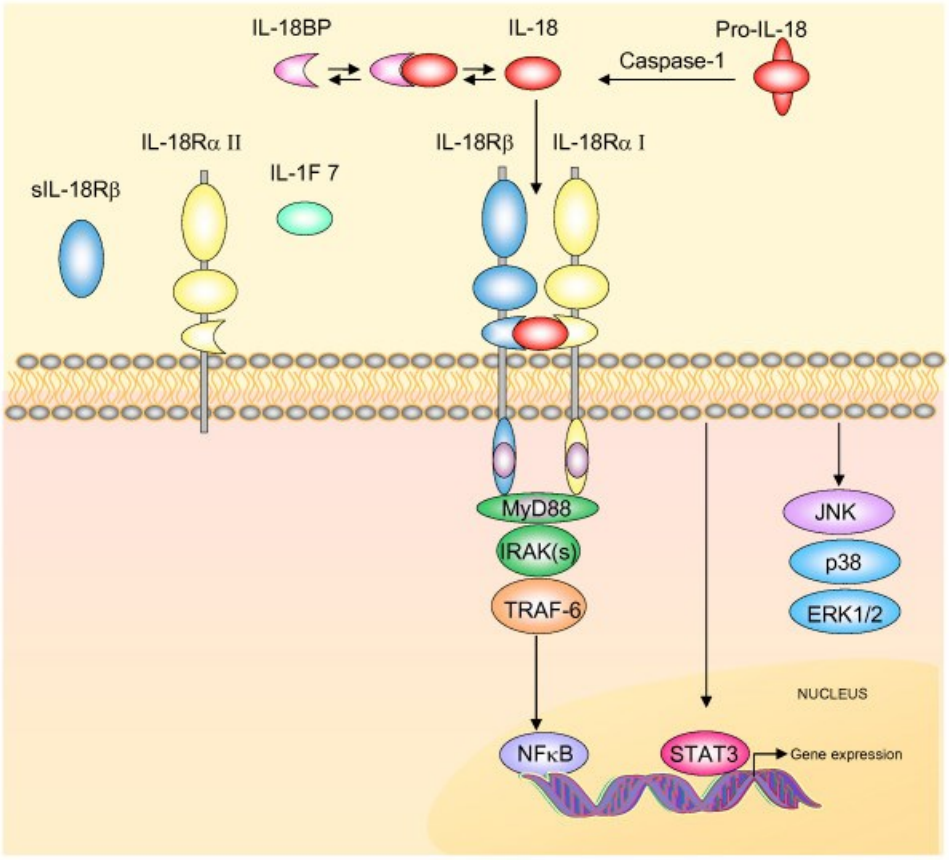

Figure 6: Interleukin-18 signal transduction.

The schema shows IL-18 signal transduction cascade. Precursor Pro-IL-18 is activated by caspase-1 to biological active IL-18. IL-18 binding protein (IL18-BP) binds to IL-18 and inhibits its function. Free IL-18 binds to IL-18Rα and IL-18Rβ, which transduce signals via the adaptor protein MyD88. This initiates IL-1 receptor activating kinase (IRAK) autophosphorylation and interaction with the TNFR-associated factor-6 (TRAF6), which induces nuclear translocation of the nuclear factor κB (NF-κB). Activation of STAT3 and the mitogen-activated protein kinase (MAPK) p38, JNK and ERK could also be initiated also by engagement of the IL-18R complex.

1.4.4 IL-18 Binding Protein

IL-18 binding protein (IL-18BP) is the natural antagonist of IL-18. IL-18BP binds with IL-18 and prevents its recruitment to the IL-18R. Mouse IL-18BP shares around 65% of the characteristics of human IL-18BP. IL-18BP is detected in many organs in human, such as PBMCs, small intestine, thymus, and ovarian tissue [183, 184]. In the mouse, it is found in lung, heart, and placenta [185]. In the serum of healthy individuals, IL-18BP levels are normally 20 fold higher than IL-18 [186]. Four isoforms (a, b, c, and d) exist in humans and two isoforms (c and d) exist in the mouse. Although IL-18BP b and d isoforms are not capable of neutralizing IL-18, isoforms a and c have high affinity to bind and deactivate IL-18 [187]. The detection of human IL-18BPb and IL-18BPd cDNA in a monocyte library suggests a role for these isoforms in IFN-γ regulation during inflammation [188].

IL-18BP is induced and upregulated by IFN-γ, thus establishing a negative feedback mechanism. It is detectable both in vitro and in vivo [189]. *Paulukat et al*. demonstrated that epithelial cell lines express IL-18BP after treatment with IFN-γ, and that indicates the self-limitation of IL-18 functions [190]. This concept is clinically supported with data obtained from a hepatitis patient treated with IFN-α who demonstrated high serum production levels of IL-18BP [191]. In this regard, IL-27, which has both pro and anti-inflammatory characteristics, can also induce IL-18BP by feedback mechanisms [192]. IL-18BP serves some protective functions against diseases, such as collagen-induced arthritis and contact hypersensitivity [193, 194]. Neutralizing IL-18 by injecting IL-18BP in BALB/c mice protects the animals against adriamycin nephropathy, and results in less frequent proteinuria and kidney dysfunction [195]. The same protective effect can be observed by neutralizing IL-18 by IL-18BP in cases of renal ischemia reperfusion injury and liver disease [196, 197].

1.4.5 IL-18 as an Immunoregulatory Cytokine

Although IL-18 does not induce fever or prostaglandin production, the cytokine promotes inflammation by various mechanisms [198, 199]. The importance of IL-18 as an immunoregulatory cytokine can be summarized by its ability to induce IFN-γ and FasL.

Furthermore, according to the presence of other cytokines, IL-18 can participate in Th1, Th2, and Th17 response depending upon the cells milieu [**Figure 7**]. In the presence of IL-12 or IL-15, IL-18 can induce IFN-γ from various types of cells that participate in Th1 responses. In the absence of IL-12 or IL-15, IL-18 loses its capacity to induce IFN-γ, but initiates its role in Th2 diseases [181]. IL-18 knockout mice weigh 2–3 times more than wild-type mice [200]. This finding reveals the role of IL-18 in energy homeostasis. Regardless, IL-18 is necessary for IL-23 signaling and functions synergistically with IL-23 to initiate potent Th17 responses [126]. Blocking IL-18 in an experimental model of autoimmune encephalomyelitis has therapeutic benefits [201]. A similar effect is noted in animal models with other Th17 diseases like arthritis and heart disease [202, 203]. On the other hand, induction of FasL is a unique property of this multifunctional cytokine. IL-18 increases expression of FasL in cases of liver injury by inducing Fas-dependent hepatocyte apoptosis [204]. Furthermore, IL-18 mediates proapoptotic signals in renal tubular cells by increasing FasL expression. siRNA knockdown of FasL gene expression in these cells markedly decreases IL-18-induced apoptosis [205]. A previous report from our laboratory has shown that IL-18 induces FasL in the NK cell line NK92 as well as in primary human NK cells [206].

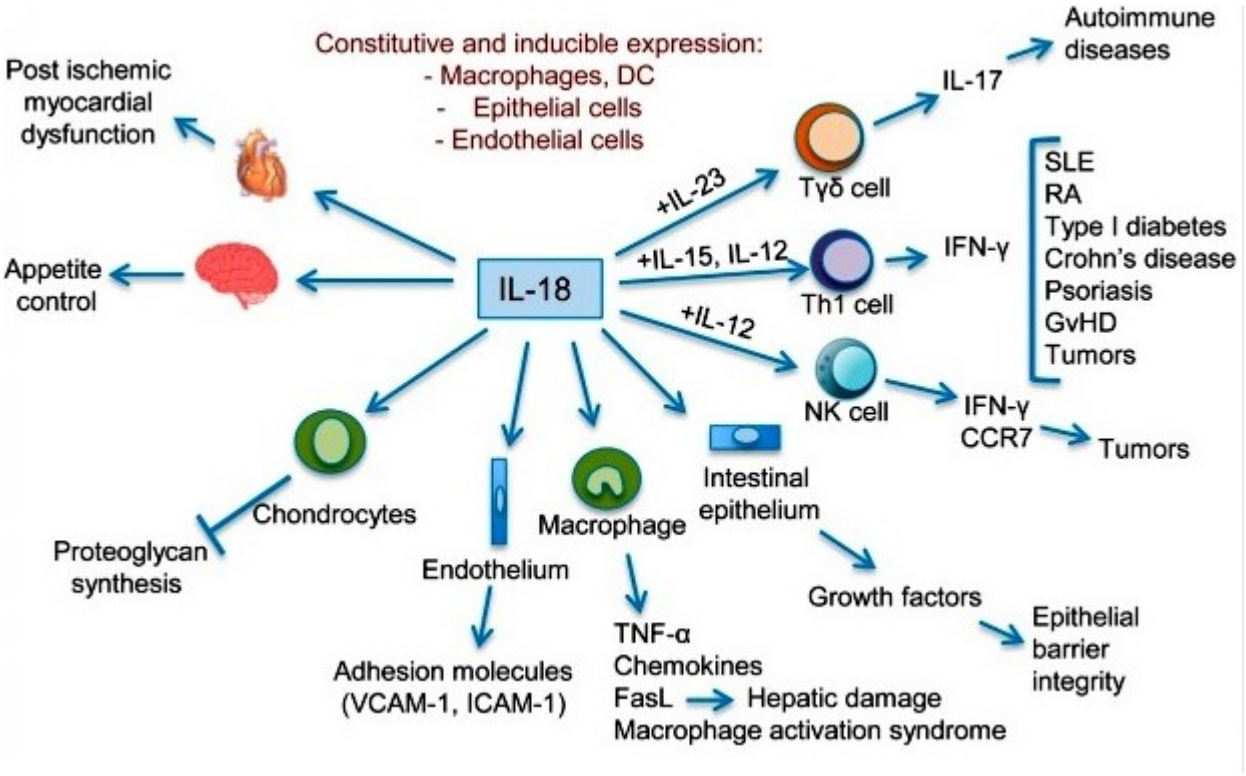

Figure 7: IL-18 in Innate and Adaptive Immunity.

Different biological effects of IL-18 and clinical conditions in which increased IL-18 activities have been implicated. SLE, systemic lupus erythematosus; RA, *rheumatoid arthritis;* GVHD, *graft-versus-host disease;* VCAM-1, vascular cell adhesion molecule 1*;* ICAM-1, intercellular adhesion molecule 1.

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1.4.6 IL-18 in Intestinal Inflammation

IBD is a chronic intestinal inflammatory autoimmune disease characterized by inflammation of the small intestine and colon and associated with macrophage infiltration at inflammatory sites. The main types of IBD include Crohn disease and ulcerative colitis, which are classified according to the nature and location of the inflammation [207]. The role of proinflammatory cytokines in IBD has been extensively studied over the past two decades [208], and animal models and human clinical data present the important role of IL-18 in inducing intestinal inflammation. Overexpressed IL-18 mice are associated with exacerbated colitis and are characterized by marked infiltration of mucosal macrophages [209]. High levels of IL-18 mRNA were demonstrated in colon cells in a dextran sulfate sodium (DSS)-induced mouse model of colitis. Neutralizing IL-18 with IL-18BP inhibits IBD and the inflammation induced by DSS in these mice [210]. To highlight the role of IL-18 in Crohn disease, IL-18 was neutralized by IL-18BP in two different murine colitis models induced by DSS and 2, 4, 6-trinitrobenzene sulphonic acid (TNBS). Also, IL-18 appears to play a role inducing the inflammatory cascade in Crohn disease [211]. Evaluation of IL-18 serum levels in patients with IBD or Crohn disease reveal an upregulation of IL-18 compared with healthy individuals [212]. Freshly isolated mucosal lymphocytes from patients with Crohn disease, but not from healthy individuals, demonstrate significant proliferative responses to IL-18 [213]. Furthermore, immunohistochemical analysis of lamina propria mononuclear cells (LPMC) and intestinal epithelial cells (IEC) isolated from patients with Crohn disease showed greater localization of IL-18 in macrophages, DCs, and IECs compared with healthy individuals [212]. IL-18BP is also upregulated in submucosal intestinal macrophages and endothelial cells of patients with Crohn disease, but this increase in IL-18BP is due to inactive (b and d) rather than active (a and c) isoforms [214]. Interestingly, IL-18 may play a protective role during intestinal inflammation by restoring homeostasis and facilitating tissue repair. This concept is supported by the observation that IL-18 KO mice develop more severe DSS-induced colitis [342]. Although an increase of IL-18 level in the circulation is a signal for inflammatory diseases, the cytokine plays an essential role in inducing an immune response and protecting against infection. It also plays

a role in wound healing and tissue repair. The fundamental physiological effects of this cytokine are dependent upon its levels as well as upon the cytokine mileu.

There are conflicting data supporting the role of IL-18 in inflammation associated with GI-related cancer. IL-18 appears to offer a protective role against cancer by activating NK cells and inducing tumor cell death and regression [215]. Nude mice injected with the cancer cell line Bacp37 following transfection with or without the IL-18 gene demonstrate a loss in Bcap37 cell tumorigenicity due to IL-18 gene engineering [216]. In contrast, elevated levels of IL-18 in serum and tissue tend to be associated with human gastric carcinoma [217]. The role of IL-18 in promoting metastasis has also been described [218].

1.4.7 IL-18 and HIV

IL-18 has been well studied in the pathogenicity and infectivity of HIV on account of its multifunctionality. As described previously, IL-18 has proapoptotic effects and induces IFN-γ from NK, T, and NKT cells, and initiates Th1 and Th2 responses depending on the context. High serum levels of IL-18 are present in recently infected HIV patients, and this increase is associated with increased viral load in untreated patients [219, 220]. HAART successfully reduces these high levels of IL-18 [221]. Earlier published work produced by our laboratory demonstrated the existence of high serum levels of IL-18 in primary and chronic infected patients, and in viremic and aviremic patients treated with HARRT compared with healthy individuals. In the same study, we found that serum concentrations of the IL-18 antagonist, IL-18BP, were significantly decreased in these HIV-infected patients compared with noninfected subjects, as summarised in [**Figure 8**] [222]. As described previously, IL-18BP is predominantly induced by IFN-γ, which is produced by IL-12, IL-15, or IL-18. The finding that IL-12 and IL-15 are decreased in HIV infection provides a plausible explanation for the decrease of IL-18BP despite the presence of high levels of active IL-18 [223, 224]. Furthermore, increased expression of IL-18 and decreased expression of IL-18BP in monocyte-derived macrophages after HIV infection in vitro supports previous in vivo findings [222]. Similar investigations with animal model revealed similar findings. Generating infection in monkeys by pathogenic SIV/HIV-1 (SHIV) produces high levels of IL-18. This elevation of IL-18 was accompanied by higher viral load and rapid loss of CD4+ T cells [225]. Elevated IL-18 during HIV infection has been

proposed as a biomarker for acute activation of T cells and as an indicator of disease progression [226].

Recent genetic studies report a link between IL-18 polymorphism and increased risk of HIV infection. Data generated from 500 HIV-1/AIDS patients and 500 age- and gender-matched controls revealed that IL-18 -137 G allele and G/G genotype appear to be implicated in the pathogenesis of HIV-1[227]. Elevated IL-18 in HIV infection has many biological effects that result in increased progression of infection and the induction of AIDS. Some of these effects will be summarized in the following paragraphs.

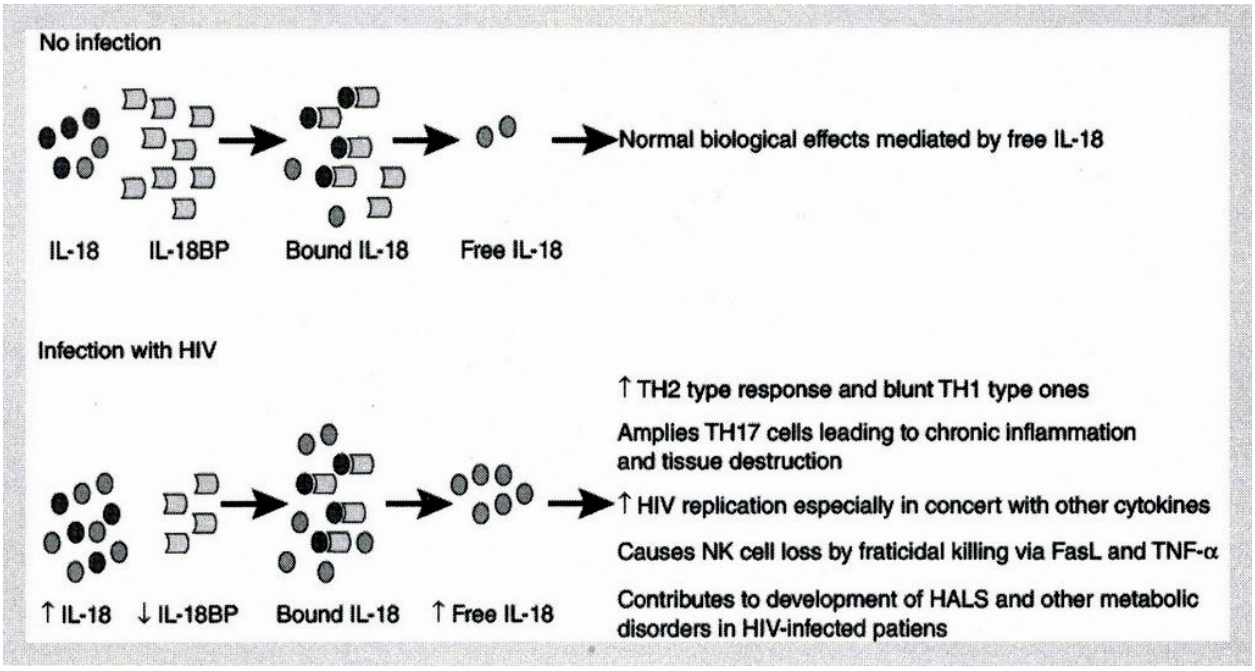

Figure 8: Imbalance between IL-18 and IL-18BP production during HIV infection.

Potential influence of IL-18 in HIV pathogenesis. Up regulation of IL-18 and down regulation of IL-18BP production were associated with progression of HIV infection. Increased IL-18 in circulation contributes towards inflammation and AIDS development. HALS, HIV-associated lipodystrophy syndrome.

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1.4.8 The Role of IL-18 in HIV-associated Disorders

Lipodystrophy and hyperlipidemia are very common and frustrating HIV-associated abnormalities. The association between HIV and these abnormalities was first described in 1998 [228]. Redistribution of body fat is reported in the majority of people suffering from lipodystrophy and hyperlipidemia. *Empty cheek syndrome* and *buffalo hump condition* are two examples of fat redistribution in HIV patients. In empty cheek syndrome, patients lose subcutaneous fat from the face region, while in the buffalo hump condition, patients gain more fat in the dorsocervical region. This redistribution of fat can also occur in the arms, legs, trunk, breasts, and viscera [229]. Unfortunately, these syndromes are exacerbated by the use of HAART [229]. A role for IL-18 in these HIV-related syndromes has been proposed since high levels of IL-18 occur during HIV infection. Transcripts for IL-18 and its receptor occur in adipose tissue and inducing adipocytes [230]. Prevention of acute lipolysis by acipimox or insulin results in decreased adiponectin and IL-18 levels in patients with HIV-associated lipodystrophy [231]. Although IL-18 levels are high in HIV, IL-18 levels are further increased in patients with lipodystrophy [232]. In addition, the level of IL-18 gene expression in patients with HIV-associated lipodystrophy further correlates with the degree of lipotrophy [233]. In a Brazilian genetic study, single nucleotide polymorphisms (SNPs) at positions -607(C/A) and -137(C/G) in the promoter region of the IL-18 gene were examined in HIV-infected patients suffering from lipodystrophy (n=88), HIV-infected patients without lipodystrophy (n=79), and healthy controls (n=133). Interestingly, the 607A allele, -607AA genotype, and -137G/-607A and -137C/-607A haplotypes were over represented in HIV patients suffering from lipodystrophy [234].

Increased triglycerides and insulin resistance are two other common findings associated with combined ART in HIV infection [235, 236]. Several studies have shown a central role for IL-18 in initiating insulin resistance, with a recent report showing that IL-18 serum levels have substantial positive associations with insulin resistance markers [237]. Insulin resistance develops gradually as a result of obesity-induced inflammation [238]. High levels of cytokines induce inflammatory mediators, such as TNF-α and IL-6, and correlate with HIV-associated insulin resistance [239]. IL-18 levels also appear to correlate with diabetes mellitus and coronary

artery disease (CAD), and patients with both conditions exhibit higher IL-18 levels than patients with diabetes mellitus alone or CAD alone [240]. However, the precise mechanisms by which IL-18 induces insulin resistance and diabetes mellitus are yet to be elucidated. IL-18 also positively correlates with acute pancreatitis and destruction of insulin-producing cells in both animal models and hospitalized patients [241, 242]. Interestingly, neutralizing IL-18 in a mouse immunoinflammatory diabetes model by daily administration of IL-18BP for 7 days revealed a lower incidence of diabetes and milder insulitis compared with untreated mice [243]. Similar results were demonstrated in NOD mice; neutralizing endogenous IL-18 with IL-18BP resulted in a significant reduction in the cumulative incidence of diabetes [244]. An increase in pancreas size was observed in IL-18 knockout mice [245]. Regardless, further investigations are necessary to define the role of IL-18 in inducing insulin resistance and diabetes mellitus, since these observations strongly suggest that IL-18 mediates insulin resistance in HIV-infected patients.

A comparative analysis for 13 cohort studies examining the cause of death in HIV-1 infected patients have shown that about 6.5% of AIDS-related mortality is attributable to cardiovascular disease [246]. Triglyceride, total cholesterol, and low-density lipoprotein cholesterol levels are elevated in 70% of HAART-treated patients [247]. Since higher concentrations of IL-18 occur in patients with CAD than in healthy individuals [240], IL-18 level is considered an essential predictive marker in coronary disease and cardiovascular mortality [248, 249]. On the other hand association between hypertriglyceridemia and elevated IL-18 in HIV-infected patients highlights the role of IL-18 in HIV-associated cardiovascular disorders [250]. The role of IL-18 in atherosclerosis induction during the early stages of HIV infection was evaluated in an experimental monkey model of SIV. The results strongly imply a role for IL-18 in early atherosclerosis progression during the infection, and the authors postulated a role in accelerating atherogenesis in AIDS patients [251].

HIV infects the central nervous system (CNS) during early asymptomatic infection and the virus continues to cause nerve damage when left untreated [252]. HIV-associated neurocognitive disorders like HIV-associated dementia (HAD) have been reported in many patients. However, the prevalence of dementia in HIV varies between 5% and 20% depending

on the response to HIV treatment [253, 254]. In the CNS, IL-18 is expressed by activated microglia, astrocytes, ependymal cells, and neurons [255, 256]. Moreover, IL-18 may modulate neuronal excitability [257]. IL-18 regulates anxiety and fear memory in an experimental mouse model [258]. In humans, IL-18 is detected in the CSF of patients with HAD but not in healthy individuals [259]. Taking these observations into consideration, it seems that IL-18 plays a role in inducing HAD and other CNS conditions during HIV infection.

Chapter 2

HYPOTHESES, RATIONALE, OBJECTIVES & AIMS

We hypothesized that platelets could be a source of IL-18 in the course of HIV infection, and that the cytokine plays a role in disturbing intestinal integrity in HIV-infected individuals.

Our overall objective was to have a better understanding of the potential role of IL-18 in the immunopathogenesis of AIDS. We wished to achieve this objective by testing our hypotheses. Accordingly the specific aims of the research work described in the thesis were:

1. To investigate whether platelets contribute to increased concentrations of IL-18 in HIV-infected individuals, and
2. To investigate the potential role of the cytokine in affecting integrity of the intestinal tract.

RATIONALE:

The rationale for hypothesizing platelets as a potential new source of IL-18 was based upon two facts: 1) a previous study from this laboratory had shown a significant positive correlation between circulating cytokine concentrations and platelet activation in HIV-infected individuals [260] and 2) platelets have been well-documented to express/contain a large number of soluble mediators (both pro- and anti-inflammatory ones) and secrete them upon activation, e.g., TGF-β, IL-1β, soluble CD40L, etc; [261]. Therefore, we designed experiments to investigate whether human platelets contain/produce IL-18 and/or its antagonist (IL-18BP), and whether there is any difference between HIV-seronegative healthy and HIV-infected individuals with respect to this function of platelets.

The rationale for hypothesizing that IL-18 may contribute towards AIDS pathogenesis by increasing intestinal permeability and causing increased microbial translocation is that the cytokine is produced by intestinal epithelial cells [262]. It is noteworthy that most of HIV infections occur through mucosal surfaces [54]. In this regard, intestines represent the anatomical sites where massive replication of the virus occurs [8]. Furthermore, the intestinal barrier disturbance is a well described condition associated with HIV pathogenesis [47]. It is also known that pro-inflammatiory cytokines like IL-1β and INF-γ, etc, cause disruption of the intestinal epithelial barrier function [61, 69]. Given these facts, we hypothesized that the virus and/or its proteins may be interacting directly with the intestinal epithelial cells (IEC) and induce secretion of this cytokine. Given that pro-inflammatory cytokines belonging to the IL-1Family disrupt integrity of inter-epithelial cell junctions, and increase intestinal permeability via

paracellular spaces [70, 263], it is quite conceivable that the virus-induced IL-18 from IEC may increase intestinal permeability, increase microbial translocation and activate immune system.

Chapter 3

The chapter contains a research article that describes our investigations on the first aim: to investigate whether human platelets contain/express IL-18 and/or its antagonist, and if so, then how the infection affects their ability to produce these soluble mediators.

The manuscript is formatted for submission to Blood for publication.

Manuscript 1

Differential synthesis and release of IL-18 and IL-18 Binding Protein in human platelets, and their implications for HIV-infected AIDS patients

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Running Title: Expression of IL-18 and IL-18BP in human platelets

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Key Points:

1. Human platelets synthesize IL-18 de novo upon activation but contain pre-formed IL-18BP.
2. They release them with different kinetics contributing towards their imbalance in the circulation.
3. The secretion of IL-18, but not of IL-18BP, is inhibited by glyburide.

Author Contributions: AA, OA and SS designed the research plan, OA performed the experiments, OA and SS analyzed data, and OA wrote the first draft of the manuscript. All other authors read, contributed to, and approved the manuscript.

ABSTRACT

IL-18 is a pro-inflammatory cytokine belonging to the IL-1 family and is produced from macrophages, dendritic cells, keratinocytes, and adrenal cortex. The cytokine is produced as a leaderless inactive precursor that is cleaved into its mature form by activated caspase-1. The caspase itself exists as an inactive precursor in human cells, and requires assembly of an inflammasome and activation of caspase-1 for its maturation. We show here that human platelets contain transcripts for IL-18. They synthesize the cytokine de novo and release it upon activation. The platelets also contain inflammasome components, which assemble and activate caspase-1 during the platelet activation process. Platelets also contain and release IL-18-Binding Protein (IL-18BP), a naturally occurring IL-18 antagonist; however, it is not synthesized in them de novo. The two soluble mediators are secreted from platelets with different kinetics. The secretion of IL-18, but not of IL-18BP, is inhibited by glyburide, an inhibitor of inflammasome assembly. We also show here that the platelet-released IL-18 constitutes the major source of this cytokine in the circulation of healthy individuals. Unlike healthy individuals, the platelet-poor plasma from HIV-infected individuals contains significant amounts of this cytokine. Our findings have important implications for viral infections and other human diseases that are accompanied by platelet activation.

INTRODUCTION

IL-18 is a pro-inflammatory and multifunctional cytokine belonging to the IL-1 family [1-3]. Like IL-1β, a prototypic member of the family, IL-18 is produced as a leaderless 24 kD precursor, which is cleaved by caspase-1 into a mature and biologically active 18 kD protein [4]. The cytokine is produced by a variety of cell types and tissues in the body and exerts many immune regulatory activities. It acts in synergism with IL-12 to induce INF-γ from NK and T cells (reviewed in [2, 3]). It increases cytolytic potential of NK cells and CD8+ T lymphocytes, and induces expression of FasL in these cells. It promotes proliferation and development of TH1 type CD4+ T cells. However, in the absence of IL-12, it promotes IgE production and the development of TH-2 type CD4+ T cells [5] . In more recent studies, IL-18 and related cytokines were shown to play an essential role in the development of TH17 cells [6] . In addition to its immune regulatory effects, IL-18 exerts profound metabolic effects and plays an important role in energy homeostasis by affecting appetite and energy expenditure [7, 8] . Increasing IL-18 concentrations in the circulation acts as an independent risk factor for insulin resistance, atherosclerosis, and cardiovascular diseases [9-11]. Not surprisingly, the cytokine has been implicated in the pathogenesis of several chronic inflammatory and autoimmune diseases [12-15].

The biological activities of the cytokine are controlled by a naturally occurring IL-18 antagonist called IL-18BP [16, 17]. The antagonist, produced in the body constitutively and as a negative feedback response to IL-18 bioactivity, binds and inactivates IL-18 with high affinity.

Platelets (thrombocytes) are small discoid anucleate cells that arise from megakaryocytes [18, 19]. Their role in hemostasis and thrombosis is well established. However, it is increasingly being realized that they also play an important role in inflammation, immune regulation and energy homeostasis. They do so by releasing a variety of immunologically important molecules including TGF-β, IL-1-β, RANTES, MIP-1α, CD40L and Thromboxane A2, etc. as well as via direct contact with other cells [20, 21]. Platelets are known to become activated in viral infections and chronic inflammatory conditions. Previously, we showed a significant positive correlation between circulating IL-18 concentrations and platelet activation in HIV-infected individuals [22], which implied that platelets may secrete this cytokine upon activation. Here we tested this implication and provide direct evidence for the first time that human platelets synthesize and secrete IL-18 de novo upon activation.

MATERIALS AND METHODS

Platelet Preparations

Human platelet preparations were obtained from the peripheral venous blood as described earlier [23, 24]. Briefly, the blood was obtained from donors in 8.5 ml glass tubes (Vacutainer, Becton Dickinson) containing 1.5 ml of the Acid-Citrate-Dextrose (ACD) buffer. For the platelets that were used as none-activated controls, an equal volume of 4% paraformaldehyde (PFA) in PBS (pH 7.20) was added to the blood immediately after collection. The samples were centrifuged at 150 g for 15 minutes and the platelet-containing plasma was obtained. Platelets were obtained from the plasma by centrifugation at 1000 g for 10 minutes. The supernatant was kept as platelet-poor plasma (PPP). After washing twice, the pellets were gently re-suspended in PBS ($5x10^8$ per ml). The preparations contained fewer than three thousand nucleated cells per ml, and fewer than 5% expressed CD63 on their surface (data not shown). To quantitate IL18 and IL-18BP in platelet lysates by ELISA, the platelets were subjected to three consecutive cycles of freeze and thaw followed by sonication for 20 seconds on ice.

Differentiation of K562 into megakaryocytes

K562 are leukemic cells that can be differentiated in vitro into platelet precursor cells, megakaryocytes [25]. For this purpose, 1 million cells were incubated with PMA (10 ng per ml; from Sigma-Aldrich) and a P38 MAPK inhibitor, SB202190 (2 uM concentration; from Sigma-Aldrich) for 72 hours as described [25, 26].

Reagents

Thrombin, TGF-β, ADP, Escherichia coli O55:B5 LPS, Brefeldin A, glyburide and cycloheximide were from Sigma-Aldrich. ATP was from Amersham Biosciences and collagen from Chrono-Log. Caspase-1 inhibitor (Z-YVAD-FMK) was from Santa Cruz. Their used final concentrations are indicated in individual experiments. To treat platelets with different reagents, the reagents were added in 10 ul volume to 100 ul of the platelet preparations. After incubation at room temperature for 30 minutes, the platelet pellets and supernatants were obtained from the

preparation by centrifugation at 1000 g for 4 minutes. In some experiments, platelets were pre-incubated with cycloheximide for 10 minutes for blocking protein synthesis.

Western blots

To investigate protein expression, platelet pellets were lysed by re-suspending them in the lysis buffer followed by sonication for 20 seconds on ice as described earlier [27, 28]. Western blots were performed on proteins precipitated from the platelet supernatants using Trichloroacetic acid (TCA). The lysates were clarified by centrifugation for 15 min at 14,000 g and equal amounts of the lysate proteins (40-50 ug) were resolved on 12% SDS-PAGE under reducing conditions. The resolved proteins on the gel were electroblotted onto PVD membranes (Immobilon; Millipore). The unbound sites on the membranes were blocked with 1X casein solution (Vector Labs). The IL-18 protein bands were detected by incubating membranes with IL-18 rabbit polyclonal antibody from MBL (catalogue # PM014). For detection of IL-18BP, goat anti-human IL-18BP antibodies from R&D (catalogue #AF 119) were used. Protein bands were revealed by enhanced chemiluminescence (Vectastain ABC-AMP; Vector Laboratories, AK-6601). The blots were photographed by the Gene Genius Bio-Imaging System (Syngene).

Co-immunoprecipitation

In some experiments, ASC, the Apoptosis-associated Speck-like protein containing Caspase activation and recruitment domain (CARD), and caspase-1 were co-immunoprecipitated from activated and non-activated platelet lysates. It is noteworthy that activation of caspase-1, required for processing and release of mature IL-18 from human cells [4], occurs upon assembly of a multi-protein complex called inflammasome. During this assembly, ASC oligomerizes and recruits the caspase-1 precursor, which undergoes auto-cleavage to become biologically active caspase-1 ([29]; reviewed in [30]). The interaction between the two proteins (caspase-1 and ASC), determined by their co-immunoprecipitation, is considered as a test for the assembly of an inflammasome in cells [29]. To determine their co-immunoprecipitations, antibodies specific for ASC (mouse anti-human ASC from MBL, catalogue # D086-3) and for caspase-1 (rabbit anti-human caspase-1 from Cell Signaling Technology, catalogue # 2225S) were used. The antibodies were cleared off stabilizing proteins and were coupled covalently to agarose resin beads using kits from Pierce (Antibody Clean-up kit Catalogue # 44600 and Co-

Immunoprecipitation Kit; Catalogue # 26149) and used as per manufacturer's recommendations. The eluted proteins were mixed with the sample buffer, boiled with 1 uM DTT and analyzed by Western blotting under reducing conditions as described in the above section. The proteins, co-immunoprecipitated with anti-ASC antibody, were tested for the presence of caspase-1 and vice versa on the Western blots.

Measuring IL-18 and IL-18BP concentrations

The concentrations of IL-18 and IL-18BP were determined in serum, plasma and platelets lysates by commercial ELISA kits obtained from eBioscience and R&D Systems, respectively. The lower detection limits of the kits were 78 pg/ml and 100 pg/ml, respectively.

Determining steady-state mRNA levels

The presence of transcripts for IL-18, IL-18BP, IL-8 and GAPDH were determined in platelets by RT-PCR. Total RNA was isolated from platelet pellets ($1x10^9$) using RNeasy Mini Kit from Qiagen (Catalog No. 74106). cDNA was made by reverse transcribing 0.5 ug RNA obtained from the platelets with a two-step RT-PCR kit from Qiagen (QuantiTect Reverse Transcription Kit Cat. No 205311. The reaction was carried out in a total volume of 20 ul with 30 minutes' incubation at 42°C. The cDNA from the transcripts was amplified by PCR using published gene-specific primers (Table 1; [31-33]). We also performed RT-PCR for the transcripts of GAPDH and IL-8 genes as positive and negative controls on cDNA from platelets, since the transcripts for these two genes have been shown to be present and absent, respectively, in human platelets [34, 35]. 5-10 ul of each PCR reaction was analyzed for the expected size DNA band by electrophoresis on 1% agarose gel. The DNA bands were revealed by ethidium bromide and photographed using the Gene Genius Bio-Imaging System (Syngene).

Patients

Peripheral blood was obtained for preparation of serum and platelets from 7 HIV infected patients from local AIDS clinics after their written informed consent. All the patients were naïve to anti-retroviral therapy and had the following demographic and clinical characteristics: age, 22-54 years (median 34); CD4+ T cells, 165-1033 per ul (median = 527.8); viral load (log10

values), 4.09 to 5.04 RNA copies per ml (median =4.57); platelets counts, 154 to 332 million per ml (median = 230.83).

Staining for intra-cellular cytokines

Activated and non-activated platelets were stained for intracellular IL-18, IL-18BP and F-actin, and were examined under a fluorescent microscope. Briefly, platelets were spread on Poly-L-lysine coated glass slides and either treated with thrombin or 4% PFA for 30 minutes. They were fixed and permeabilized by incubation on ice in equal volumes of methanol and 4% PFA. The slides were incubated with IL-18 rabbit polyclonal antibody (MBL; catalogue # PM014), goat anti-IL-18BP antibodies (R&D; catalogue #AF 119) or control antibodies for 1 hour at room temperature. After washing, the slides were incubated for 1 hour at room temperature with relevant fluorochrome-conjugated secondary antibodies (FITC-conjugated mouse anti-goat IgG from e-Bioscience; or with Alexa Fluor® 488-conjugated goat anti-rabbit IgG from Life Technology). The slides were subsequently washed, stained with TRITC-conjugated Phalloidin (Sigma-Aldrich) and examined under the Eclipse E800 microscope (Nikon).

Confocal microscopy

The differentiated K562 cells were attached to glass slides using poly-L-lysine, fixed with 4% PFA in PBS and permeabilized with Triton X-100. The cells were incubated with anti-human IL-18 or human IL-18P (catalog numbers PM014 and AF119, obtained from MPL and R&D, respectively), followed by washing and staining with FITC- or PE-conjugated goat anti-human IgG (both from eBioscience). After washing, the cells were stained with PE-conjugated anti-human CD63 (from eBioscience) or with FITC-conjugated anti-human CD62 (from eBioscience). The stained cells were imaged with a deconvolution system (Deltavision RT; Applied Precision) using an inverted microscope (1×70; Olympus) with a 100× 1.4 NA oil-immersion objective and a cooled charge-coupled device camera (Coolsnap HQ; Photometrics). The Z sections were taken 0.25 μm apart. To deconvolute the resulting datasets, Softworx software (Applied Precision) was used and maximum intensity projections were saved as TIFF files as described earlier [36].

Statistical analysis

Means were compared using student's "t" test between two groups. For multiple comparisons, ANOVA was performed followed by comparison of means using Tukey's test.

RESULTS

Activated human platelets express IL-18

To determine whether human platelets expressed IL-18, we obtained lysates from thrombin treated platelets as well from PFA-treated inactivated platelets. We performed Western blots on these lysates for the expression of the cytokine. As shown in Figure 1A, human platelets expressed both precursor and mature forms of the cytokine. Interestingly, un-activated platelets (treated with PFA) expressed neither of the two forms of the cytokine. We detected β-actin from PFA-treated cells suggesting that the PFA treatment did not affect the Western blot results. Nevertheless, it was possible that the treatment may have inhibited detection of IL-18 on Western blots. Therefore, we activated platelets with thrombin for 30 minutes, and prepared lysates with and without treatment with PFA. As shown in Figure 1B, the PFA treatment did not interfere with the detection of IL-18 in thrombin-treated platelets. Essentially similar results were obtained from five different donors (data not shown).

It is well known that platelets can be activated by a variety of different reagents. We were interested in determining if any of the reagents exerted any differential effect on the production of IL-18 in platelets. Figure 2A shows that irrespective of the activating reagent, activated platelets expressed this cytokine, albeit at different levels.

In order to determine whether upon activation platelets release IL-18 into the medium, we collected their supernatants and well as cell pellets by centrifugation. The cell pellets were also re-suspended in an equal volume of PBS, frozen and thawed three times and sonicated. The concentrations of the cytokine were determined in the supernatants and lysates by ELISA. As shown in the Figure 2B, the platelets released the cytokine into the medium upon activation. Figure 2C shows total amounts of IL-18 (in lysates plus supernatants) produced from differentially treated platelets. Consistent with the data in Figure1B and 2A, the platelets fixed with PFA showed significantly ($p<0.01$) reduced quantities of the cytokine in lysates, supernatants and total amount compared with the platelets activated with different treatments (Figure 2B and C).

De novo synthesis of IL-18 in activated platelets

The lack of IL-18 protein in PFA-treated platelets suggested its de novo synthesis in activated platelets. To confirm this, we sought to determine the effect of a translation inhibitor

cycloheximide [37, 38] on the production of the cytokine in activated human platelets. As shown in Figure 3A, the cytokine was detected in the lysates of activated platelets but was markedly decreased when the cells were activated in the presence of cycloheximide. Interestingly, no effect of the cycloheximide treatment was seen upon expression of β-actin and IL-18BP (see below) in the platelets. The cytokine concentrations were also markedly decreased in the supernatants when the platelets were activated in the presence of the inhibitor (Figure 3B). Furthermore, IL-18 was readily detectable by immunofluorescence upon intracellular staining in activated platelets but not in non-activated ones (Figure 3C).

Activated platelets contribute to IL-18 concentrations in the plasma

If activated platelets were the main contributors of IL-18 present in the circulation in healthy individuals, then freshly isolated platelet-poor plasma (PPP) would lack this cytokine. To test this notion, we isolated PPP and determined its IL-18 contents by ELISA. At the same time, we collected serum from the donors. The Figure 4 shows data (mean ± SD) from 5 different donors. It is noteworthy that the PPP collected immediately after the blood collection from the donors contains relatively little IL-18 compared to their plasma and serum samples. These data suggest that platelet-released IL-18 is the main contributor to the cytokine concentrations present in the plasma in healthy donors. In line with these results, the plasma from the PFA- treated blood also contained significantly less concentrations of the cytokine compared to the plasma without the fixative (Figure 4). Furthermore, it is noteworthy that serum consistently contained less cytokine concentrations as compared with their autologous plasma, although overall differences between their means were not significant ($p>0.05$).

Activation of caspase-1 in platelets

The expression of mature IL-18 in activated platelets implies that caspase-1 becomes activated in these cells. To determine this, we performed Western blots on platelet lysates obtained from PFA-treated and thrombin-activated human platelets. As shown in Figure 5A, the PFA-treated non-activated platelet lysates contain only the precursor form, whereas the activated platelet lysates also contain the activated form of the caspase-1. We also measured activation of caspase-1 by using the caspase-1/ICE colorimetric assay kit from BioVision (cat #k111-25) following

the manufacturer's recommendations. The results (Figure 5B) show that the caspase-1 is activated in platelets upon activation with thrombin but not in PFA-fixed platelets.

Platelets assemble inflammasome upon activation

Caspase-1 activation suggests assembly of an inflammasome in platelets upon activation. To determine whether inflammasome assembly occurs in activated platelets, we sought to determine whether immunoprecipitation of ASC results in co-immunoprecipitation of caspase-1 and vice versa. As shown in Figure 6, caspase-1 can be detected in the Western blots performed on the ASC immunoprecipitate and vice versa in activated platelet lysates but not in PFA-treated non-activated platelets.

Existence of IL-18 transcripts in human platelets

De novo synthesis of IL-18 suggests the presence of IL-18 transcripts in platelets. To investigate it, we isolated total RNA from human platelets, reverse-transcribed it and used IL-18 cDNA-specific primers, and determined on agarose gels whether they yielded amplified DNA fragment of the expected size. The data shown in Figure 7 suggests that human platelets contain transcripts for IL-18 and GAPDH, but not for IL-8 and IL-18BP genes. The transcripts for these two genes were readily amplifiable from the cDNA prepared from the human PBMC (Figure 7).

Human platelets express IL-18BP

Because of the importance of IL-18BP in neutralizing IL-18, we sought to determine whether platelets also express it. For this purpose, we determined its expression in their platelet lysates by Western blots. As shown in Figure 8A, activated and PFA-treated non-activated platelet lysates expressed the protein. The data suggest that unlike IL-18, IL-18BP is present in both activated and non-activated platelets, and hence is not synthesized de novo upon activation. In support of this notion, we did not detect any effect of cycloheximide on the expression of IL-18BP in platelets (Figure 3A). Furthermore, the RT-PCR on platelet-derived RNA for IL-18BP gene did not yield the DNA band of the expected size (Figure 7). Collectively, these data suggest that IL-18BP is present pre-formed in platelets, and is not synthesized de novo upon activation. In line with these results, and contrary to the ones seen with IL-18, the concentrations of IL-18BP were more or less similar in PPP and plasma from the same individual (Figure 8E)

We also determined intracellular expression of IL-18BP in human platelets with and without activation by immunofluorescence. As shown in Figure 8B, both activated and non-activated human platelets expressed IL-18BP intracellularly.

In order to determine the release of IL-18BP from platelets in response to different platelet-activating agents, we isolated platelets from five different donors', activated them with different activators and measured IL-18BP in the platelets lysates and supernatants by using a commercial ELISA kit. Figure 8C depicts mean ± SD of the IL-18BP concentrations in the supernatants. It is noteworthy that different activators released different amounts of the protein in the supernatants. Total IL-18BP produced in these differentially treated platelets is shown in Figure 8D. Interestingly, non-activated platelets produced more IL-18BP in both lysates and supernatants, which is consistent with the results obtained from Western blots shown in Figure 8A.

We measured IL-18BP concentrations in the plasma, plasma from PFA-added blood, PPP, and serum from five different donors. Figure 8E depicts mean ± SD values of the protein found in these compartments. Unlike IL-18, whose levels were minimal in PPP as well as in the plasma from the PFA-treated blood, PPP had relatively higher levels of the IL-18BP. These data suggest that unlike IL-18, IL-18BP is constitutively present in the circulation of healthy individuals and platelets are very likely to contribute towards its levels in the circulation, as non-activated platelets constitutively release it and do not require platelet activation.

We performed a kinetic study and determined the release of IL-18 and its antagonist over time from activated platelets. IL-18 secretion continued to increase in the culture supernatant at least until 6 hours post-activation, whereas no increase was observed in the release of IL-18 BP from the platelets over this time. In fact, IL-18BP release was significantly ($p<0.01$) decreased within an hour after activation (Figure 9).

Differential secretion of IL-18 and IL-18BP from platelets

It is noteworthy that like IL-1β, IL-18 lacks a leader peptide and follows a non-conventional pathway of secretion. This kind of secretion is effectively inhibited by glyburide [39]. On the other hand, IL-18BP has a leader peptide and follows the classical secretory pathway [40]. Therefore, we sought to investigate the effects of glyburide on the secretion of these two soluble mediators from platelets. The results are shown in Figure 10 A and B. The treatment prevented processing of the precursor IL-18 into its mature form, and also prevented its secretion into supernatants. On the other hand, glyburide had no effect on the expression and secretion of IL-18BP. As expected, caspase-1 inhibitor (Z-YVAD-FMK) pretreatment decreased the expression of mature IL-18 in platelet, and had no effect on that of IL-18BP. Brefeldin A inhibited the secretion of both IL-18BP and mature IL-18 from activated platelets.

Colocalization of IL-18 and IL-18BP with different cellular marker

Using intracellular staining and confocal microscopy, we determined the expression and colocalization of IL-18 and IL-18BP with two different cellular markers in platelet precursor cells (megakaryocytes) differentiated *in vitro* from K562 cells. The markers, CD62P and CD63, localize to α-granules and lysosomes and⁄or dense granules, respectively in platelets [41]. As shown in Figure 11, IL-18BP colocalized with CD62P, but not with CD63, whereas IL-18 colocalized mainly with CD62P and to a much lesser extent with CD63.

Implications for HIV-infected individuals

Our results show that IL-18 released from activated platelets contributes to its levels in the circulation in healthy individuals. This implies that IL-18 may be present in increased concentrations in the circulation and PPP in HIV-infected individuals that are accompanied by platelet activation ([42]; reviewed in [43]). Therefore, we sought to determine concentrations of IL-18 and its antagonist in platelet lysates, PPP and plasma samples vis-à-vis their healthy control counterparts. As shown in Figure 12A, the concentrations of the cytokine were less in platelet lysates from HIV-infected individuals. PPP from HIV-infected donors contained an average of 75 pg/ml of the cytokine whereas the PPP from healthy donors contained the cytokine below the detection limit of the kit. As for IL-18BP, we detected lower concentrations of the antagonist in the platelet lysates from HIV-infected individuals compared with those in the healthy control subjects, although the differences were not statistically significant ($p>0.05$;

Figure 12B). Similarly, mean concentrations of this soluble mediator in PPP were not statistically different ($p<0.05$) between healthy controls and HIV-infected individuals. However, a significantly ($p<0.001$) lower concentration of the antagonist was detected in the plasma samples from the virus-infected individuals compared to the healthy control donors.

DISCUSSION

To the best of our knowledge, this is the first report describing the production of IL-18 and its antagonist from human platelets. IL-18 is synthesized de novo upon activation whereas IL-18BP is present pre-formed in these cellular elements. These conclusions were reached as the platelets,

which were isolated from blood that was immediately treated with PFA to fix platelets and hence prevent their activation, did not express IL-18. These data suggest that quiescent platelets do not contain pre-formed IL-18 but they rather synthesize it de novo upon activation. To confirm it, we pre-treated platelets with cycloheximide, a known inhibitor of protein synthesis [37, 38], before their activation with thrombin. As expected, IL-18 was markedly decreased in the lysates of the cycloheximide pre-treated platelets. These data provide direct evidence that IL-18 is synthesized de novo in these anucleate cellular elements upon activation and they do not contain or express it when in quiescence. However, it was not the case with IL-18BP. Unlike IL-18, PFA-treated platelets expressed IL-18BP. Furthermore, cycloheximide pre-treatment had no effect on the expression of this IL-18 antagonist. In line with these results, freshly isolated platelet-poor-plasma (PPP) had little IL-18 but was rich in IL-18BP. These data suggest that quiescent platelets release pre-formed IL-18BP but not IL-18. Consequently, PPP contains IL-18BP but not IL-18. The differential production and release of these two soluble mediators result from the presence/absence of some poorly understood motifs in their transcripts that may be required for their non-random incorporation into platelets [44]. Furthermore, IL-18 mRNA is has no signal sequences and is translated on free ribosomes in the cytosol as a leaderless polypeptide [3]. Such proteins are secreted independent of ER and a Golgi via a non-conventional mode of secretion [45]. This differential regulation ensures that IL-18BP is present in the circulation of an individual, as it is present in pre-formed form in platelets and its release does not require platelet activation, whereas IL-18 is only synthesized de novo and released from platelets upon activation. Since IL-18BP inactivates IL-18 via its high affinity binding to the latter [16, 40], its continuous presence in the circulation may serve as a buffer against highly pro-inflammatory systemic effects of IL-18 in the circulation, should platelets synthesize and release it into circulation upon activation. Here we add yet another pro-inflammatory cytokine, IL-18, as well as its antagonist, IL-18BP, to the growing list of soluble mediators produced/released by human platelets.

Platelets lack nuclei and genomic DNA. Therefore, a traditional mechanism of gene expression, i.e., transcription of DNA does not occur in them. The de novo synthesis of IL-18 in platelets upon activation suggests the presence of the gene transcripts in platelets. We tested this possibility and were able to observe the presence of transcripts for the cytokine gene, but not

for the IL-18BP gene, by RT-PCR performed on the total RNA obtained from the platelets. We also detected in them transcripts for GAPDH, but not for the IL-8 gene. This is the first report describing the presence of transcripts for the IL-18 gene and synthesis of IL-18 protein in human platelets upon their activation. Interestingly, a previous report described the presence of transcripts for IL-1β, a prototype member of the cytokine family to which IL-18 belongs [35]. More recently, cDNA for another proiflammatory protein, Macrophage Migration Inhibitory Factor (MIF) was shown to be present in platelets [26]. It has been shown that megakaryocytes, the precursors of platelets, transfer about 3-6 thousand distinct mRNAs to platelets in an organized, non-random fashion, which may be translated into proteins constitutively and/or upon platelet activation [46]. Our results show that IL-18 is one of those proteins.

IL-18BP and IL-18 are secreted from cells via classical and non-classical secretory pathways, respectively [47]. To investigate whether they follow their respective modes of secretion in platelets, we activated platelets in the presence of glyburide or brefeldin A (BFA), and determined their secretion in lysates and supernatants. As expected, glyburide inhibited the processing and secretion of mature IL-18, and had no effect on the secretion of IL-18. Unexpectedly, the BFA treatment inhibited the secretion of both mature IL-18 and IL-18BP into supernatants. BFA is known to inhibit the classical pathway of secretion by redistributing Golgi proteins into the ER [48]. The unconventional pathway of secretion actually occurs in several heterogeneous ways, some of which involve the formation of membrane-bound vesicles [49]. As BFA inhibits vesicular trafficking by inhibiting recruitment of ADP-ribosylation factor (ARF)-1 to the cell membranes [50], our results suggest that the unconventional pathway of secretion adopted by IL-18 involves this process. More studies are required to further understand this process.

With regard to the kinetics of secretion of IL-18, we noted that activated platelets continued to increase the production/release of the cytokine for a several hours after their activation. The secretion of the cytokine continued until 6 hours post-activation. However, the secretion of its antagonist, IL-18BP, decreased and remained at a plateau until 24 hours. In fact, the release of the antagonist from activated platelets was always less than that released by their quiescent counterparts. The release of this antagonist decreased both in platelet lysates and in supernatants resulting in decreased total protein. The decrease occurred within an hour after activation of

platelets. The exact mechanism responsible for this decrease in IL-18BP upon platelet activation remains unknown. Further studies are required to understand the molecular mechanisms behind this phenomenon. Nevertheless, this differential release of the two soluble mediators from activated platelets bears important implications in situations where platelets become activated, e.g., viral infections. Under these circumstances, activated platelets would continue to increase the release of IL-18, whereas the release of IL-18BP would decrease and will not increase with time. Consequently, this would result in an imbalance between the cytokine and its antagonist over time as far as their production from platelets is concerned. We have recently shown that such an imbalance between IL-18 and IL-18BP is present in the circulation of patients chronically infected with HIV-1 [51, 52]. The imbalance could contribute to the pathogenesis of AIDS in a variety of ways [53]. Normally, IL-18BP protein is synthesized and released as a negative feedback mechanism in response to IL-18. The cytokine induces, in collaboration with IL-12, Interferon-gamma (IFN-g), which is essential for the induction of IL-18BP gene [32, 54]. This negative feedback mechanism is not operative in platelets, as they do not contain IL-18BP gene or its transcripts. The induction of IL-18BP gene from other body cells in response to IL-18 may be defective in HIV infected patients, as the production of IL-12 becomes compromised in these subjects [55]. IL-18 alone does not induce IFN-γ; it rather promotes TH2 type cytokine production under IL-12 deficient conditions [5]. Our study suggests that platelet activation may play at least a partial role in the imbalanced production of IL-18 and of its antagonist in HIV-infected patients.

Our co-localization studies in *in vitro* differentiated megakaryocytes suggest that both IL-18 and IL-18BP mainly localize with CD62P, which is mainly located in α-granules [41]. We observed only smaller amounts of IL-18BP with CD63, a marker of dense granules and secretory lysosomes [41]. The α-granules contain hundreds of proteins with pro-inflammatory, anti-inflammatory, angiogenic, anti-angiogenic, and immune regulatory effects. Researchers have always wondered as to how platelets control secretion of the soluble mediators with diverse and sometimes with opposite effects. Recent studies have shown that α-granules are quite heterogeneous and may have different contents [56]. Their exocytosis and hence secretion of different soluble mediators is likely to be controlled by different stimuli. It would be highly

interesting to identify these stimuli. With this knowledge, one could control the secretion of anti-inflammatory, pro-inflammatory and angiogenic mediators from platelets.

Interestingly, we found little IL-18 in PPP from healthy subjects suggesting that minimal levels of the cytokine are present in the circulation of healthy subjects. Platelet activation may represent at least one source for increased IL-18 levels in the circulation of the individuals that experience platelet activation. Unlike healthy individuals, the cytokine was present in significantly higher concentrations in PPP obtained from HIV-infected individuals. It is tempting to speculate that activated platelets represent a major source of this cytokine in the circulation of HIV-infected individuals.

We show here that upon activation human platelets can assemble inflammasomes and process precursor caspase-1 into its activated form. This is in accordance with a recent report [57]. A large variety of exogenous (bacterial toxins, asbestos, silica) and endogenous (ATP, urea crystals, reactive oxygen species) "danger/damage" signals is known to induce inflammasome assembly in human cells (reviewed in [58]). Further work is needed to identify the exact mechanism(s) involved in inflammasome assembly in activated platelets.

In summary, we show here for the first time that human platelets synthesize IL-18 de novo from the gene transcripts, process it into its active form and release it upon activation. In contrast, they contain pre-formed IL-18BP and release it constitutively. These results have important implications in disease conditions in which platelets become activated *in vivo* and increased concentrations of IL-18 play a pathogenic role.

In summary, we show here that human platelets constitute an important source of IL-18 in the body. Interestingly, the cytokine is synthesized, processed and secreted *de novo* by these blood cells. On the contrary, the cytokine antagonist, IL-18BP, is present in pre-made form in platelets. As observed in other cell types, IL-18 and its antagonist are also secreted by different mechanisms. Our results have implications for HIV infection and other chronic viral infections in which platelets become activated.

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The authors declare no conflict of interest.

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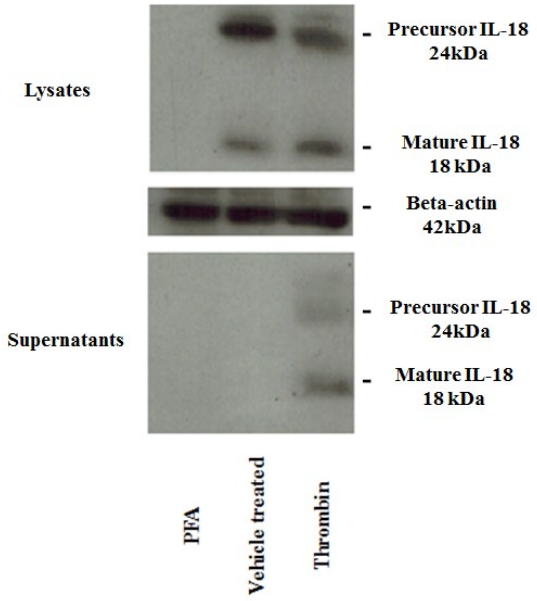
A


Lysates
Precursor IL-18
24kDa
Mature IL-18
18 kDa
Beta-actin
42kDa
Supernatants
Precursor IL-18
24kDa
Mature IL-18
18 kDa
PFA
Vehicle treated
Thrombin

B

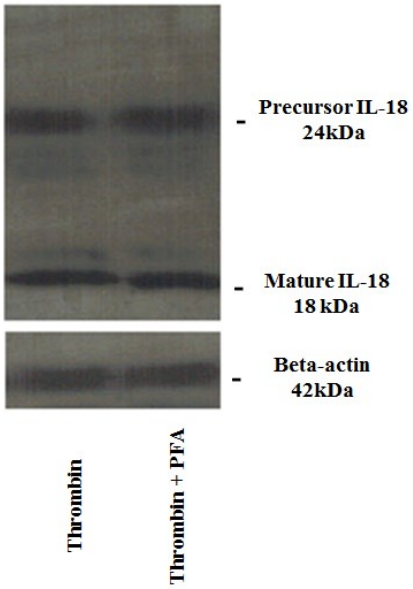
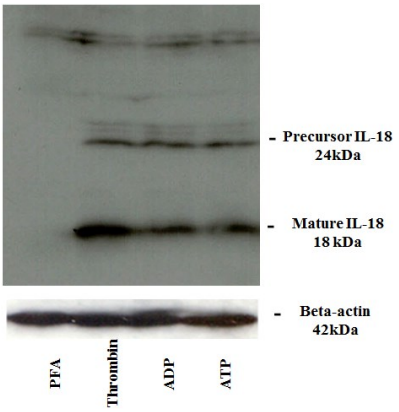

Precursor IL-18
24kDa
Mature IL-18
18 kDa
Beta-actin
42kDa
Thrombin
Thrombin + PFA

Figure 1

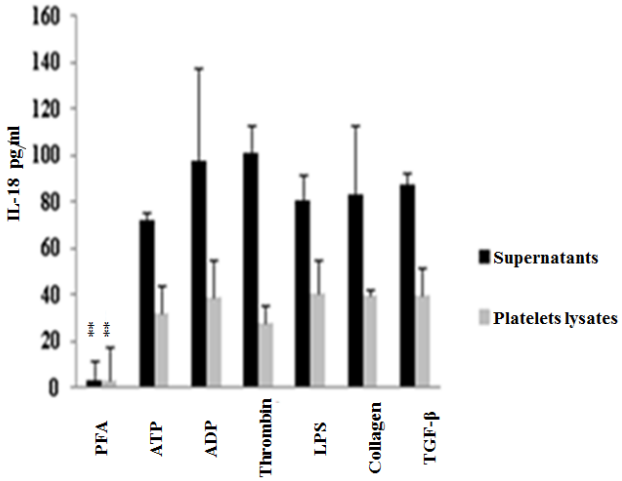
Figure 1. Human Platelets produce and release IL-18 upon activation

(A). Platelets were isolated and treated with thrombin or vehicle for 10 minutes at room temperature. An aliquot of them was fixed with PFA immediately after their isolation. After the incubation, they were centrifuged and pellets were lysed after collecting the supernatants. IL-18 bands were detected by Western blots from in the platelet lysates and supernatant. (B). To examine the effect of PFA treatment on IL-18 detection by Western blots, platelets were treated with and without PFA after 30 minutes of thrombin activation**.** The expression of the cytokine was detected by Western blots. The PFA treatment had no effect on the detection of IL-18 in Western blots.

A


Precursor IL-18
24kDa
Mature IL-18
18 kDa
Beta-actin
42kDa
PFA
Thrombin
ADP
ATP

B


IL-18 pg/ml
160
140
120
100
80
60
40
20
0
**
**
PFA
ATP
ADP
Thrombin
LPS
Collagen
TGF-β
Supernatants
Platelets lysates

C

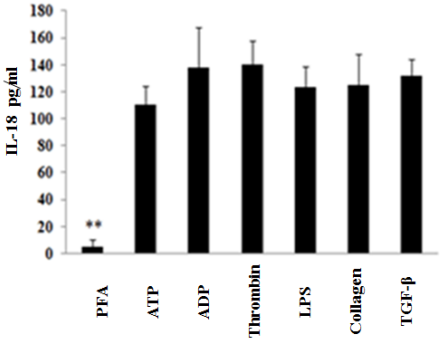

IL-18 pg/ml
180
160
140
120
100
80
60
40
20
0
**
PFA
ATP
ADP
Thrombin
LPS
Collagen
TGF-β

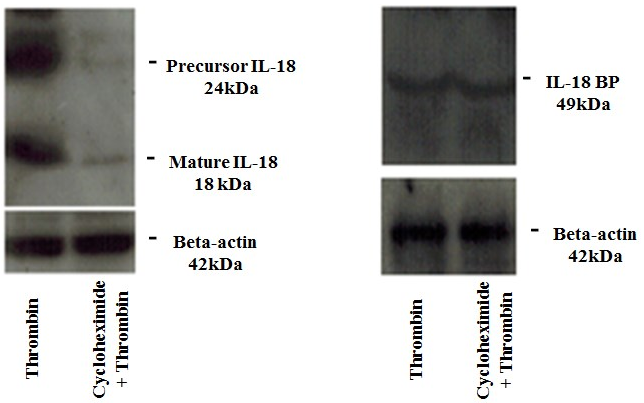
Figure 2

Figure 2. Production and secretion of IL-18 from platelets in response to different reagents

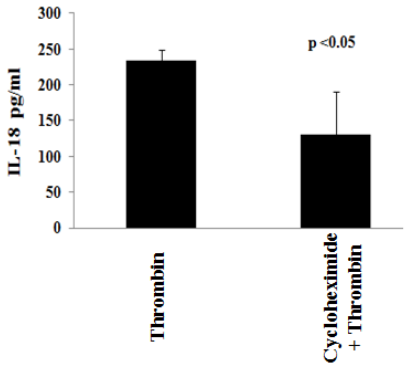
(A). Platelets were fixed with PFA or activated by different activating reagents for 10 minutes. Then IL-18 bands were detected in the lysates by Western blots. Lanes represent the lysates from platelets treated with the indicated reagents. **(B).** To determine whether platelets release IL-18 into medium upon activation with different reagents, the cytokine was measured by ELISA in the lysates and supernatants. The panel shows mean±SD of the cytokine present in the lysates and supernatants after activation with the indicated reagent. **(C).** Total IL-18 production (mean±SD from both lysates and supernatants) of the platelets in response to different treatments measured by ELISA**.**

** indicate significant ($p<0.01$) differences from other groups.

A


Precursor IL-18
24kDa
Mature IL-18
18 kDa
Beta-actin
42kDa
Thrombin
Cyclohexamide
+ Thrombin
IL-18 BP
49kDa
Beta-actin
42kDa
Thrombin
Cyclohexamide
+ Thrombin

B


IL-18 pg/ml
300
250
200
150
100
50
0
p <0.05
Thrombin
Cyclohexamide
+ Thrombin

C

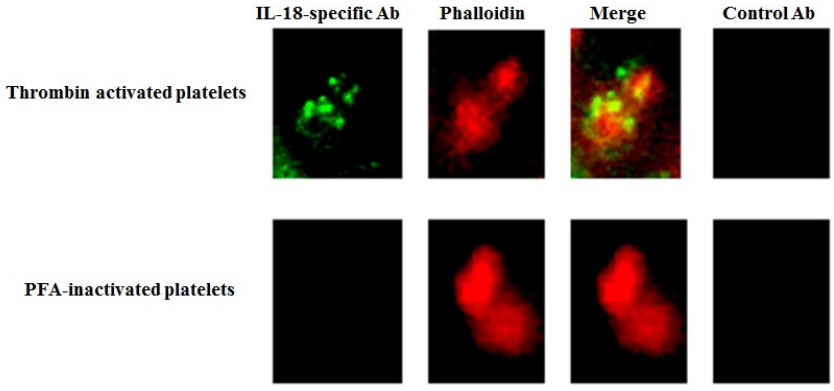

IL-18-specific Ab
Phalloidin
Merge
Control Ab
Thrombin activated platelets
PFA-inactivated platelets

Figure 3

Figure 3. Activated platelets synthesize IL-18 de novo but not IL-18BP

(A). To determine whether platelets synthesize IL-18 de novo upon activation, they were activated with thrombin for 10 minutes with and without pretreatment with cycloheximide (10 mg/ml for 10 minutes). Thereafter, IL-18 and IL-18BP were detected in the lysates by Western blots. Note that the cycloheximide treatment prevented production of IL-18 (left panel) but not of IL-18BP (right panel). **(B).** To determine the effect of cycloheximide on IL-18 release in the platelets medium, supernatants from the thrombin-activated platelets without and with pre-treatment with the inhibitor were collected and the cytokine level was measured by ELISA. The pre-treatment significantly decreased IL-18 in the supernatants. **(C).** IL-18 was detected intracellularly by immunofluorescence in thrombin activated platelets and PFA-inactivated platelets. The platelets were fixed, permeabilized and were either incubated with an IL-18 rabbit polyclonal antibody or normal rabbit IgG followed by washings and incubation with mouse anti-rabbit FITC-conjugated antibody. The F-actin was also stained with Tetramethylrhodamine B isothiocyanate-conjugated Phalloidin. The stained platelets were examined and photographed under a fluorescent microscope.

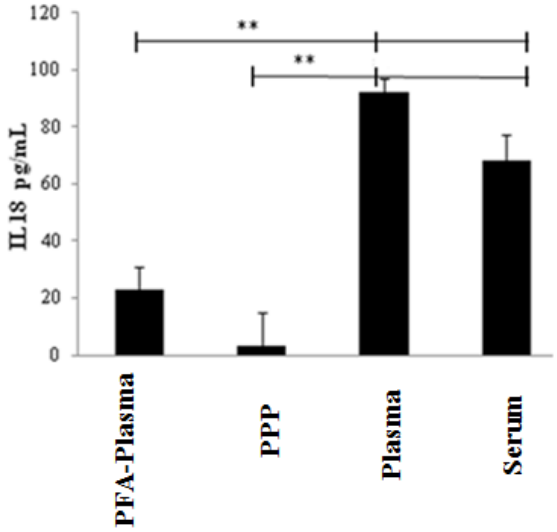
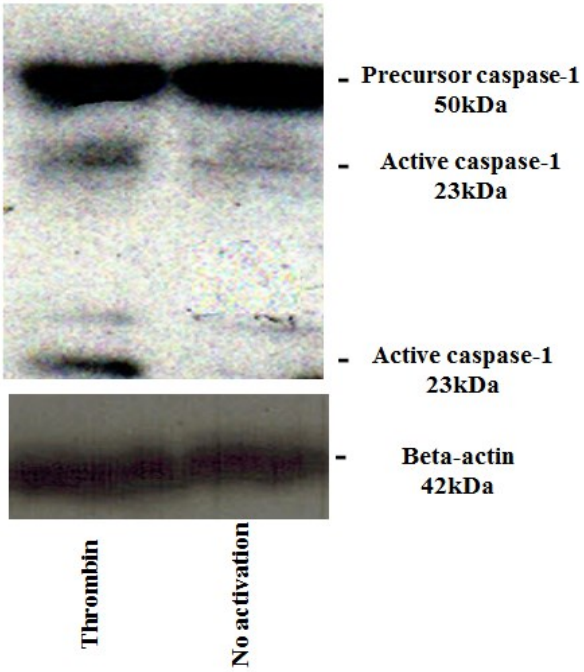


Figure 4

Figure 4. Activated platelets contribute to IL-18 concentrations in the plasma in healthy individuals

IL-18 concentrations were detected by a commercial ELISA kit. PFA-Plasma means that the plasma was obtained from the blood to which 4% PFA was added immediately after collection in equal volume. The blood from each donor (total 5) was processed to obtain PFA-Plasma, Platelet-Poor Plasma (PPP), plsma and serum. All the donors were HIV-seronagtive and healthy. The bars in the Figure depict mean ± SD. Two stars ** represents significant differences ($p<0.01$) between means.

A


Precursor caspase-1
50kDa
Active caspase-1
23kDa
Active caspase-1
23kDa
Beta-actin
42kDa
Thrombin
No activation

B

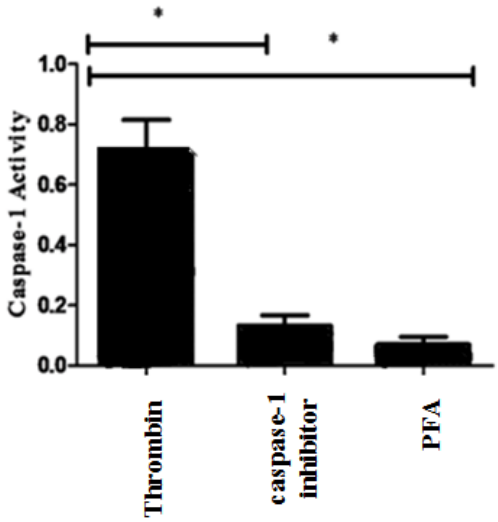

*
*
1.0
0.8
0.6
0.4
0.2
0.0
Caspase-1 Activity
Thrombin
caspase-1 inhibitor
PFA

Figure 5

Figure 5. Activation of caspase-1 upon platelet activation

(A). Platelets, with or without activation with thrombin for 10 minutes, were lyzed. Caspase-1 activation was detected by Western blots in the lysates using an antibody specific for the activated version of the caspase. Beta-actin was detected as control for protein loading. **(B).** Platelets were treated with thrombin with or without prior treatment with the caspase-1 inhibitor (Z-YVAD-FMK) for 30 minutes or PFA fixation. After washings, they were lyzed, and caspase-1 activity was measured by using the caspase-1/ICE colorimetric assay kit. The y-axis shows the caspase activity in arbitrary units.* star indicate significant ($p<0.05$) differences.

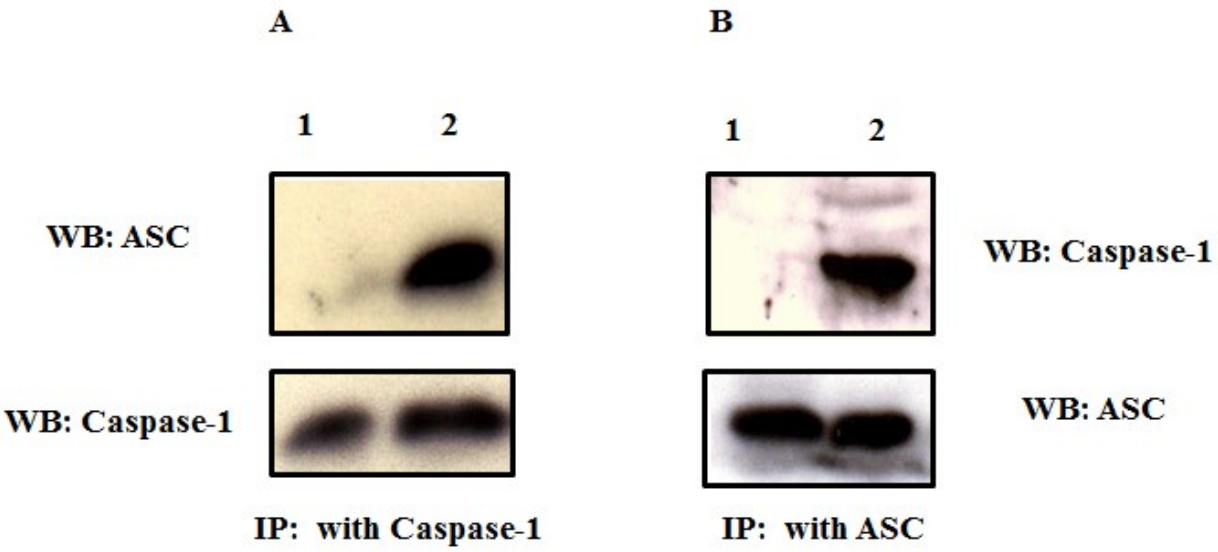

A
B
1
2
1
2
WB: ASC
WB: Caspase-1
WB: Caspase-1
WB: ASC
IP: with Caspase-1
IP: with ASC

Figure 6

Figure 6. The assembly of an inflammasome occurs in activated platelets

Caspase-1 was immunoprecipitated from non-activated PFA-fixed platelet lysates (Lane 1) and from thrombin-activated platelet lysates (Lane 2). The immunoprecipitate was subjected to Western blots for detection of ASC and caspase-1. ASC co-immunoprecipited with caspase-1 in activated platelets, but not in non-activated ones **(A)**. ASC was immunoprecipitated from these platelet lysates and the immunoprecipitate was tested for caspase-1 and ASC by Western blots. Caspase-1 co-immunoprecipitated with ASC in activated (Lane 2) but not in non-activated platelets (Lane1) **(B)**.

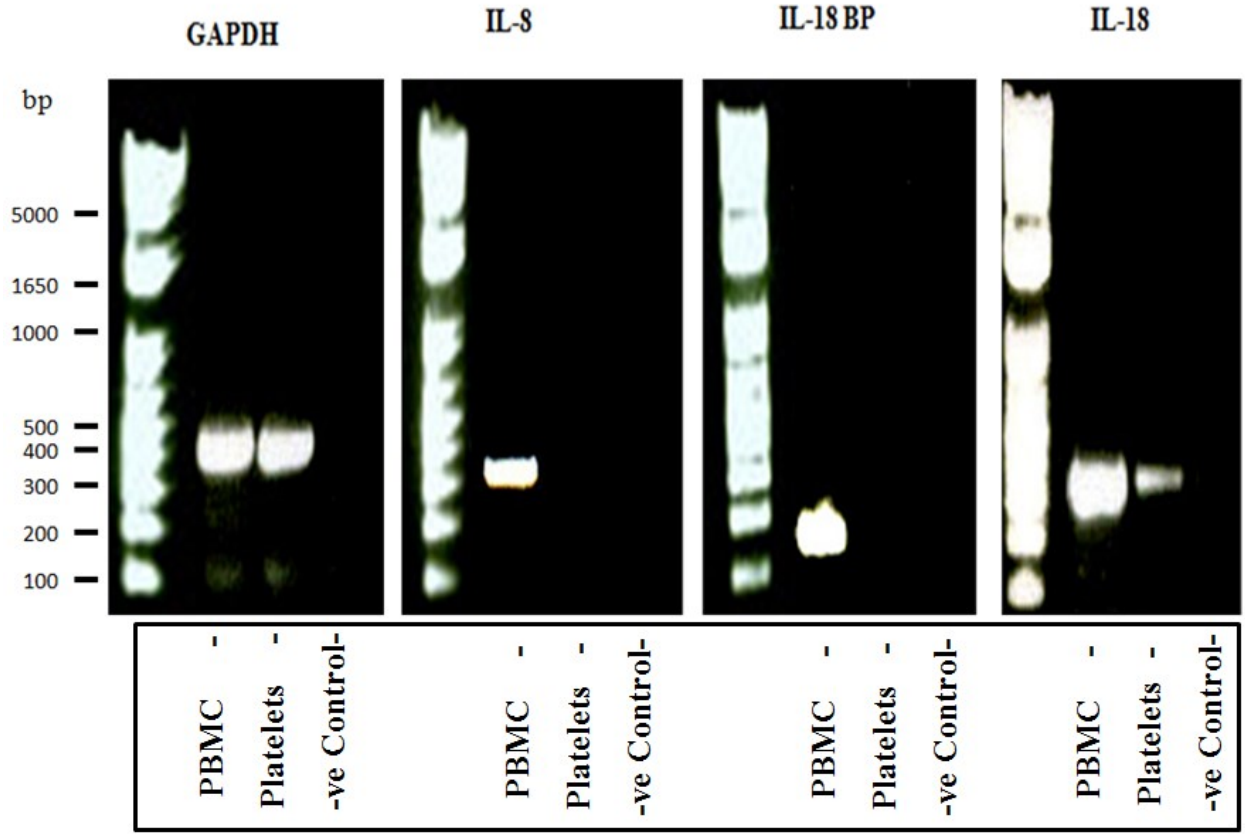
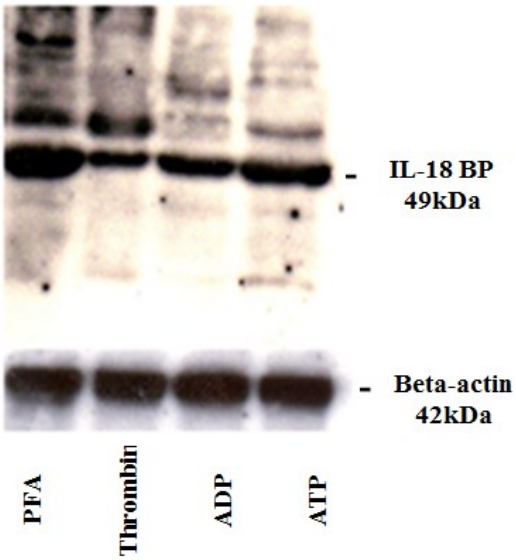

GAPDH
IL-8
IL-18 BP
IL-18
bp
5000
1650
1000
500
400
300
200
100
PBMC -
Platelets -
-ve Control-
PBMC -
Platelets -
-ve Control-
PBMC -
Platelets -
-ve Control-
PBMC -
Platelets -
-ve Control-

Figure 7

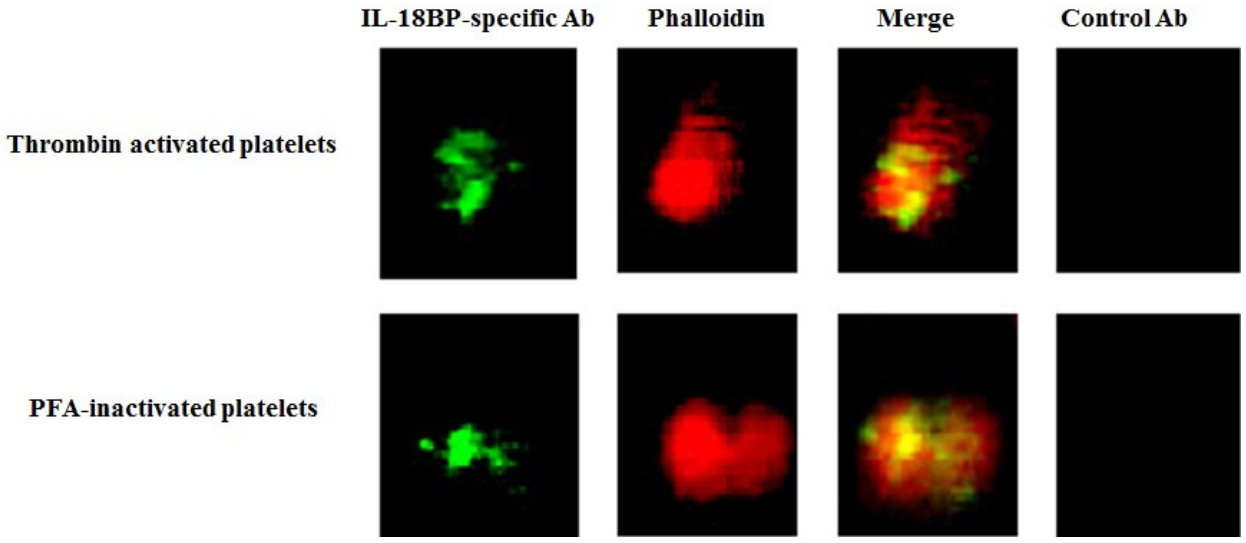
Figure 7. Platelets contain IL-18 transcripts

To determine whether platelets contain transcripts for IL-18, and other genes (IL-18BP, IL-8 and GAPDH), total RNA was isolated from platelets obtained from $3.2x10^8$ platelets and $5x10^6$ PBMC from the same donor. The RNA was reverse transcribed, and cDNA was amplified by PCR using gene-specific primers as described in the Materials & Methods' section. The presence of amplified DNA bands of the expected sizes was determined by agarose gels. The expected bands of all the genes were readily detectable in the PBMC, but the bands were detected in platelets only for IL-18 and GAPDH. No bands were detected when no template was added to the PCR reaction (-ve control).

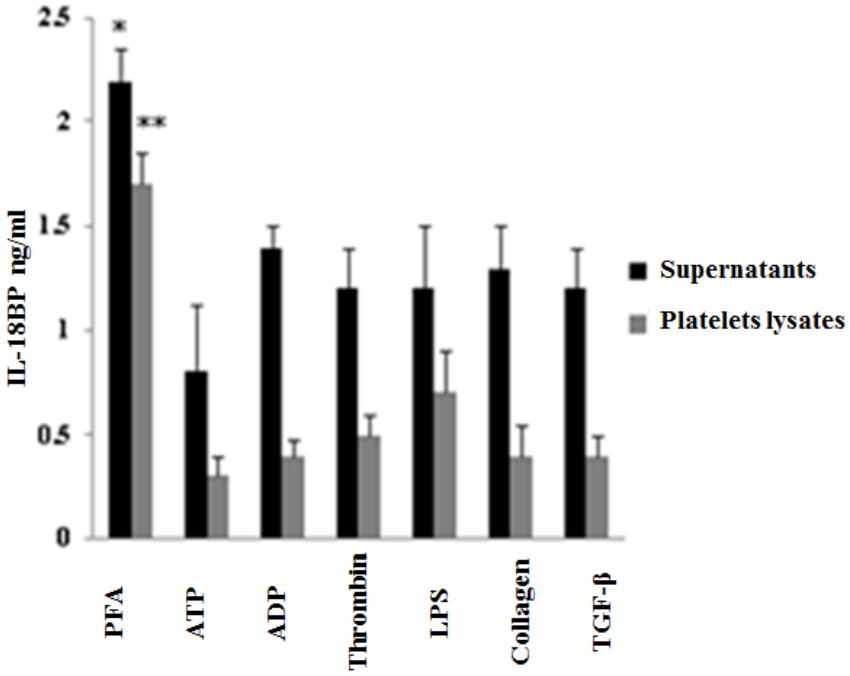
A


IL-18 BP
49kDa
Beta-actin
42kDa
PFA
Thrombin
ADP
ATP

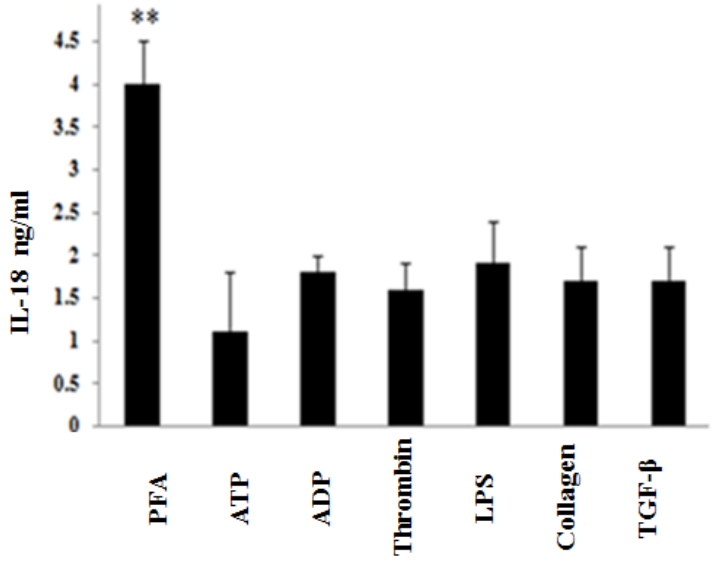
B


IL-18BP-specific Ab
Phalloidin
Merge
Control Ab
Thrombin activated platelets
PFA-inactivated platelets

C


IL-18BP ng/ml
2.5
2
1.5
1
0.5
0
*
**
Supernatants
Platelets lysates
PFA
ATP
ADP
Thrombin
LPS
Collagen
TGF-β

D


IL-18 ng/ml
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
**
PFA
ATP
ADP
Thrombin
LPS
Collagen
TGF-β

E

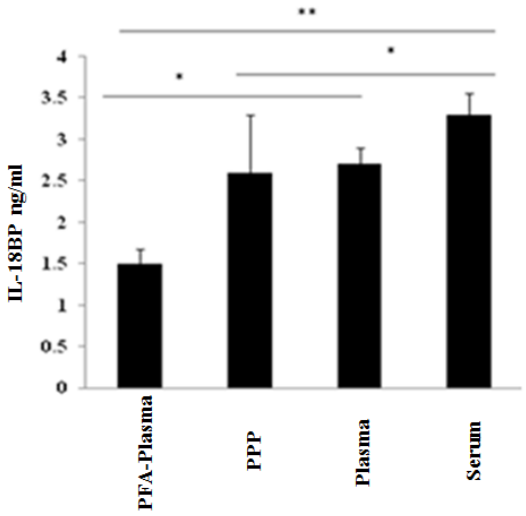


Figure 8

Figure 8. Human platelets express and release IL-18BP irrespective of activation

(A). Platelets were treated for 10 minutes with the indicated reagents, washed and lyzed. The expression of IL-18BP in the lysates was examined by Western blots. Note expression of the protein irrespective of activation. **(B).** Intracellular expression of IL-18BP in human platelets was examined after their activation with thrombin or fixation with PFA. The platelets were fixed, permeabilized and either incubated with an IL-18BP-speficic antibody or an isotype - matched control antibody followed by incubation with FITC-conjugated secondary antibodies. F-actin was stained with Tetramethylrhodamine B isothiocyanate-conjugated Phalloidin. The stained platelets were examined under a fluorescent microscope and photographed. Note expression of the protein irrespective of platelet activation. **(C).** IL-18BP released in the platelet supernatants and lysates was measured by ELISA kit after treating platelets with the indicated reagents. **(D).** Total amount of IL-18BP from lysates and supernatants as measured by ELISA. **(E).** IL-18BP concentrations in the plasma from the blood to which 4% paraformaldehyde (PFA) was added in equal volume immediately after collection, platelet-poor plasma (PPP), serum and plasma from HIV-negative healthy donors. The Figure depicts mean ± SD from five healthy donors.

In C and D panels, * indicates that values in PFA-Plasma column differ significantly ($p<0.05$) from all other columns. In panel E, * and ** indicate significant differences at $p<0.05$ and $p<0.01$, respectively, between indicated treatments.

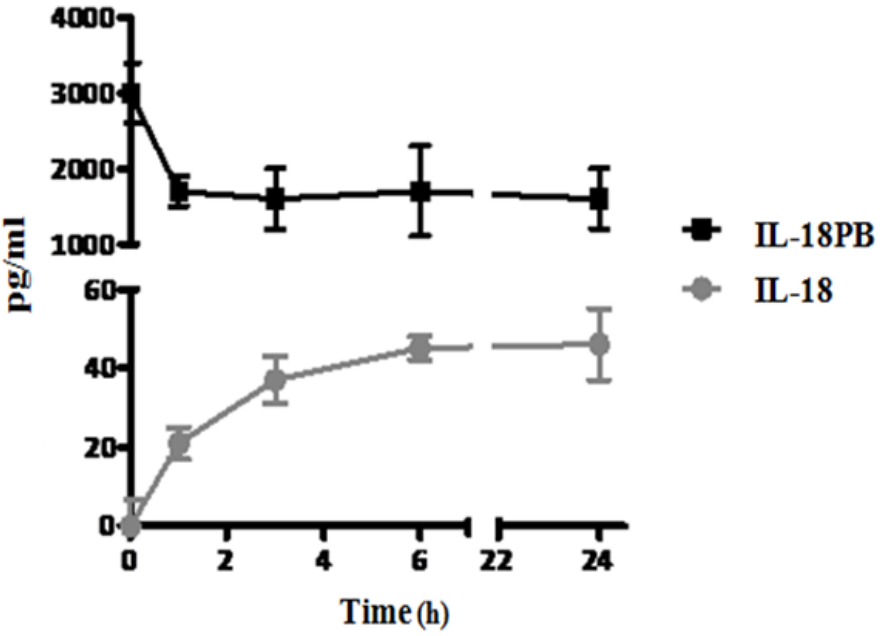
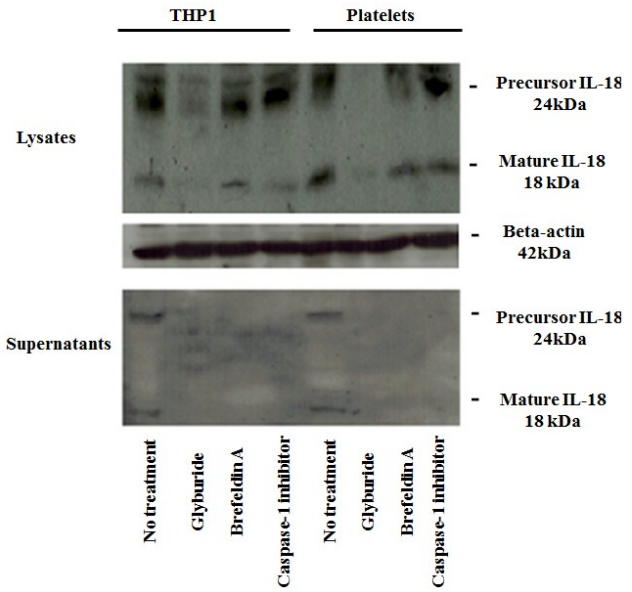

4000
3000
2000
1000
60
40
20
0
pg/ml
0
2
4
6
22
24
Time (h)
IL-18PB
IL-18

Figure 9

Figure 9. Kinetics of release of IL-18 and IL-18BP over time from activated platelets

Platelets were activated with thrombin and the concentrations of the two soluble mediators in the supernatants were determined by ELISA at indicated time points from five different donors. The Figure shows mean ± SD of IL-18 and IL-18BP at different time points. Mean values of IL-18 differed significantly ($p<0.05$) among 0, 1, 3 and 6 hour time points, and these values for IL-18BP differed significantly ($p<0.01$) only between 0 and all other time points.

A


THP1
Platelets
Lysates
Precursor IL-18
24kDa
Mature IL-18
18 kDa
Beta-actin
42kDa
Supernatants
Precursor IL-18
24kDa
Mature IL-18
18 kDa
No treatment
Glyburide
Brefeldin A
Caspase-1 inhibitor
No treatment
Glyburide
Brefeldin A
Caspase-1 inhibitor

B

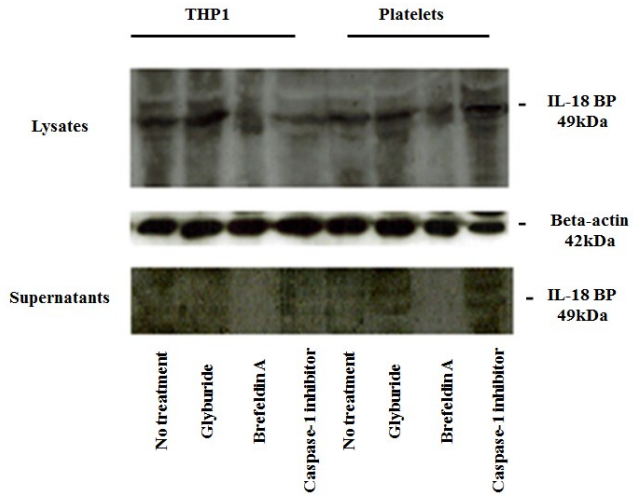

THP1
Platelets
Lysates
IL-18 BP
49kDa
Beta-actin
42kDa
Supernatants
IL-18 BP
49kDa
No treatment
Glyburide
Brefeldin A
Caspase-1 inhibitor
No treatment
Glyburide
Brefeldin A
Caspase-1 inhibitor

Figure 10

Figure 10. Different modes of IL-18 and IL-18BP secretion from activated platelets

To identify the secretory pathways of the two soluble mediators in human platelets, the latter were pretreated for 30 minutes with glyburide (400 μM), Brefeldin A (10 μg/ml), caspase-1 inhibitor (Z-YVAD-FMK; 50 μM) or with the vehicle and then activated with Thrombin (1 unit per ml). THP-1 cells were used as a control and were treated as described for platelets. Platelets lysates and supernatants were collected. IL-18 and IL-18BP were measured in them by Western blots. Western blots were performed on proteins precipitated from the platelet supernatants using TCA. Panel A and B demonstrate bands for IL-18 and IL-18BP, respectively. Beta-actin was detected as a control for protein loading both the panels.

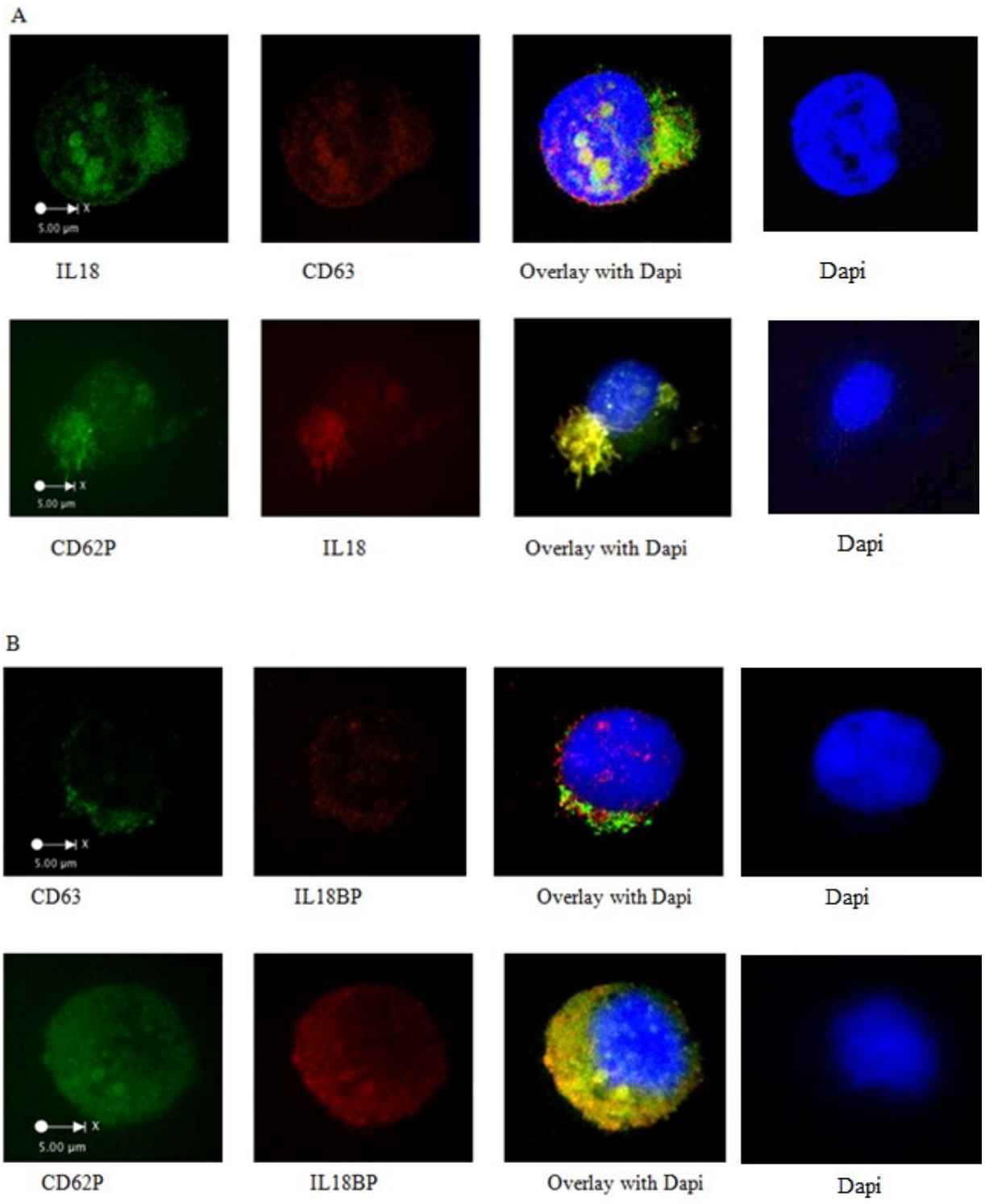
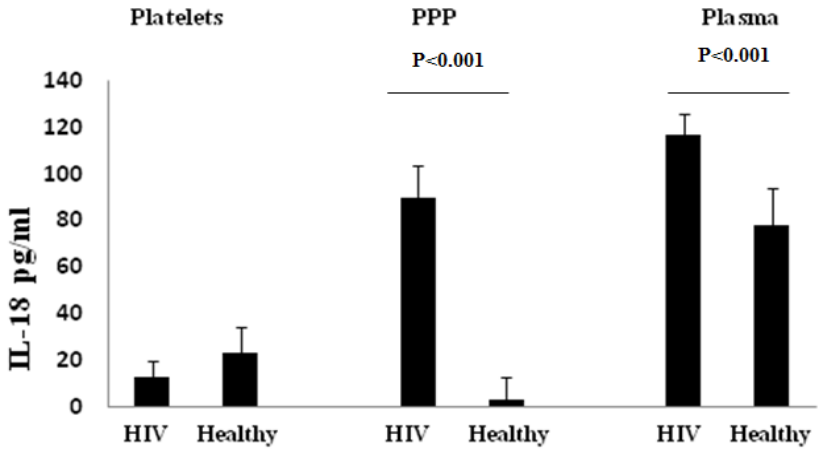

A
5.00 µm
IL18
CD63
Overlay with Dapi
Dapi
5.00 µm
CD62P
IL18
Overlay with Dapi
Dapi
B
5.00 µm
CD63
IL18BP
Overlay with Dapi
Dapi
5.00 µm
CD62P
IL18BP
Overlay with Dapi
Dapi

Figure 11

Figure 11. Colocalization of IL-18 and IL-18BP with CD62P and CD63 in platelets

Platelets precursor cells (megakaryocytes), differentiated in vitro from K562 cells, were fixed, permeabilized and incubated with anti human IL-18 or IL-18BP specific antibodies. After washing, the cells were stained with FITC-conjugated goat anti-rabbit, PE- conjugated goat anti-rabbit antibodies (for IL-18) or PE- conjugated rabbit anti-goat antibody (for IL-18BP). After the second wash, the cells were stained with PE-conjugated anti-human CD63, or with FITC-conjugated anti-human CD63. The stained cells were then counterstained with DAPI, washed and imaged with a deconvolution system (Deltavision RT; Applied Precision) using an inverted microscope (1×70; Olympus) with a 100× 1.4 NA oil-immersion objective and a cooled charge-coupled device camera (Coolsnap HQ; Photometrics). A and B depict co-localization of IL-18 and IL-18BP, respectively, with the markers in the platelet precursors.

A


Platelets
PPP
P<0.001
Plasma
P<0.001
IL-18 pg/ml
140
120
100
80
60
40
20
0
HIV
Healthy
HIV
Healthy
HIV
Healthy

B

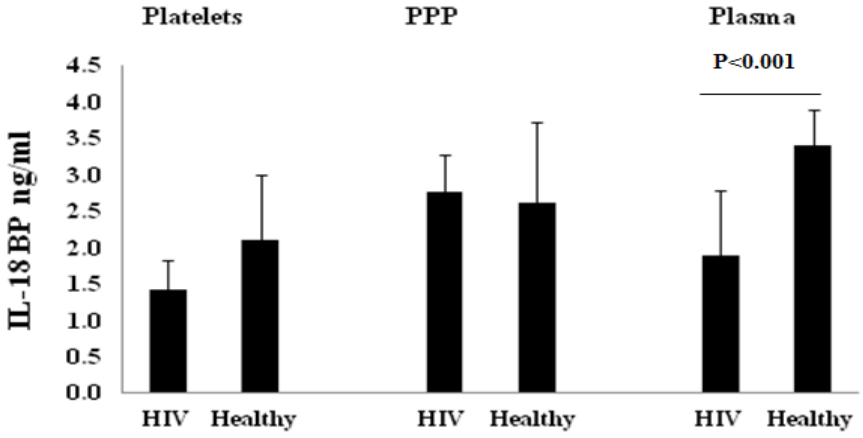

Platelets
PPP
Plasma
P<0.001
IL-18BP ng/ml
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
HIV
Healthy
HIV
Healthy
HIV
Healthy

Figure 12

Figure 12. IL-18 and IL-18BP released by platelets in healthy and HIV infected people

(A). The Figure shows concentrations (Mean ± SD) of IL-18 in platelet lysates, platelet-poor-plasma (PPP) and serum from seven HIV-infected patients and healthy control subjects as measured by ELISA. (B). The Figure depicts concentrations (Mean ± SD) of IL-18BP from same healthy and HIV infected individuals as measured by ELISA.

Chapter 4

The chapter contains our second research article that describes our investigations on the second aim, to investigate whether HIV-1 or the viral Tat protein affects production of IL-18 in human intestinal epithelial cells, and whether human recombinant IL-18 has any effect on intestinal integrity and permeability.

The manuscript is formatted for submission to the journal PLoS Pathogens.

Manuscript 2

HIV-induces IL-18 from intestinal epithelial cells that increases intestinal permeability and induces cell death

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[2] Division of Hematology & Chronic Viral Illness Service, McGill Uinversity

Running Title: HIV induces IL-18 from intestinal epithelial cells

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Author Contributions: AA, OA and SS designed the research plan, OA performed the experiments, OA and SS analyzed data, and OA wrote the first draft of the manuscript. All authors read, contributed to, and approved the manuscript.

ABSTRACT

Interleukin-18 (IL-18) has been shown to play a key role in mediating different inflammatory conditions. We and others have shown that HIV infection is accompanied by increased circulating levels of IL-18 along with decreased levels of its antagonist, IL-18 Binding Protein (IL-18BP). The infection is also accompanied by intestinal inflammation and decreased intestinal integrity as measured by mucosal repair, regeneration and permeability. However, little is known concerning the effect of HIV on IL-18 production from the intestinal epithelial cells (IEC), and the relationship between high levels of the cytokine in the circulation of HIV-infected individuals and intestinal integrity. Here we show that HIV and its protein Tat increases IL-18 production and decreases that of IL-18BP in two human IEC lines, HT29 and Caco2. IL-18 induces epithelial barrier hyperpermeability by disrupting both tight and adherens junctions, and by delocalization of occludin, claudin2 and beta-catenin. It also causes desorganization of F-actin in the IEC monolayers. Same observations were made with HIV-1 Tat protein. Moreover, IL-18 decreases transepithelial electrical resistance (TEER) in colon Caco2 and increases intestinal permeability in the intestinal HT29 cells. The cytokine induces aopotosis in IEC by activating caspase-1and caspase-3. Interestingly, the plasma levels of lipopolysaccharide (LPS) in three different categories of HIV-infected patients (ART-naïve, ART-treated and elite controllers) correlated with their IL-18 plasma levels. We found that treatment of the IEC with exogenous IL-18 upregulated myosin light-chain kinase (MLCK), phosphorylated myosin light-chain (pMLC). This increase in p-MLC was inhibited by a cell permeable Rho kinase inhibitor. The cytokine also decreased phosphorylation of STAT-5 in the IEC. Taken together our results show that HIV and Tat increase IL-18 production and decrease that of IL-18BP from IEC. The increased biological activity of the cytokine disrupts intestinal integrity and increases microbial translocation. Therefore, the cytokine plays a role in causing immune activation and promoting pathogenesis of AIDS.

INTRODUCTION

Interleukin 18 (IL-18), originally named as the interferon-γ (IFN-γ)-inducing factor, is a proinflammatory cytokine that belongs to the IL-1 family [1]. Like IL-1β, it is produced as an inactive 33 kD precursor, which is processed by caspase-1 into a mature, biologically active 17 kD form. Caspase-1 itself needs activation via assembly of an inflammasome. In the circulation, mature IL-18 is bound and inactivated by IL-18 binding protein (IL-18BP), which is produced as a negative feed-back mechanism in response to the cytokine. IL-18BP protects the body from tissue destructive effects of IL-18. IL-18 is by a wide variety of cells including, macrophages, dendritic cells, keratinocytes, adrenal cortex and epithelial cells ([2]**;** reviewed in [3, 4]). It can perform multiple context-dependent biological functions. It induces IFN-γ from NK and T cells in the presence of IL-12, and drives TH1-like immune responses. However, IL-18 promotes TH2 type responses in the absence of IL-12 by inducing IL-4 from eosinophils and naïve T cells [5]. It also induces FasL expression on NK and T cells. The cytokine has also been shown to induce death in a variety of human cells (eg, vascular endothelial and cardiac myocytes; [6]). The role of IL-18 in the inflammation of the gastrointestinal (GI) tract is controversial: IL-18 KO mice are more susceptible to dextran sulfate sodium (DSS)-induced colitis [7], whereas its concentrations are increased in the circulation of the patients suffering from inflammatory bowel disease (IBD). A strong correlation was demonstrated between IL-18 concentrations and severity of mucosal inflammation in this disease.

Previous studies from our [8] and other laboratories [9, 10] have shown that IL-18 concentrations are increased in the circulation of HIV-infected individuals. The concentrations of the cytokine increase while those of its antagonist decrease or are not correspondingly increased, resulting in increased biological activities of the cytokine. Given the fact that intestinal epithelial cells (IEC) represent an important source of IL-18, we sought to investigate whether HIV-1 has any effect on the expression and activation of this cytokine from these cell types. The issue gains more significance in view of the fact that the GI tract-associated lymphoid tissue is the primary site where HIV replicates and causes death of CD4+ T cells [11, 12]. This localized viral replication compromises intestinal barrier function. The intestines in HIV-infected individuals show 5-fold more permeability compared to their healthy counterparts. The intestinal barrier function is maintained primarily by the tight junction (TJ) proteins comprising

claudins, occludin, zona occludin (ZO)-1, 2 etc [13]. These proteins form a tight gasket between adjacent intestinal epithelial cells and regulate paracellular movement of solutes. The function of TJ is supported by another complex located underneath it called adherens junctions (AJ), which comprises E-cadherins, beta-catenin and ZO-1[14]. It has been well-documented that the intestinal barrier function is compromised in HIV-infected individuals early in the course of HIV infection, resulting in increased translocation of microbial products (e.g., LPS) in the circulation [15]. This increased translocation of microbial products is widely believed to cause increased systemic activation of the immune system in HIV-infected individuals. The resultant chronic low-grade inflammation is believed to be responsible for many AIDS-associated clinical conditions including frailty, immune senescence, accelerated aging, metabolic syndrome and lipodystrophy [16, 17]. We also addressed this question as to how IL-18 affected intestinal barrier function.

Using human IEC lines, we show here that HIV induces IL-18 production but inhibits that of its antagonist in these cell types. Furthermore, the cytokine increases intestinal permeability in the IEC monolayers and causes cell death. IL-18 may represent an important soluble mediator that promotes AIDS progression by increasing intestinal permeability and cell death.

MATERIALS AND METHODS

Epithelial Cell culture

Human colonic epithelial cells Caco2 were cultured in monolayers in Eagle's Minimum Essential Medium (EMEM; Wisent Bioproducts cat#320-012-cl) with 15% heat inactivated FBS, 2mM L-glutamine and antibiotics [18] in 37°C, 5% CO_2 and 90% relative humidity. Caco2 cells were used in experiments between passages 16-24. Human colon adenocarcinoma grade II cell line HT-29 was cultured in monolayers with McCoy's medium [19] with 10% FBS and antibiotic as described for $Caco_2$ cells. HT-29 cells were used between passages 9-19. The culture media were changed every three days and the cells were sub cultured when the monolayers reached 80-90% confluence. For sub culturing, the monolayers were by treated with trypsin-EDTA from (Sigma-Aldrich) or Versene 1X from life technologies REF 15040-066 for apoptosis assay experiments, as it is less harsh for cells than the trypsin-EDTA.

Western blot

Expressions for different proteins were investigated in the two intestinal epithelial cell lines Caco2 or HT-29. Cells were suspended in the lysis buffer containing Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), and cocktail of protease inhibitors with concentration of 1µl/0.1ml of lysates, from Sigma-Aldrich (Catalog number P8340, Oakville, CA).The cells were lysed by sonication for 20 seconds. The lysates were clarified by centrifugation for 15 min at 14,000 *g* at 4°C. The lysate proteins were resolved on 10% or 12% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions as described [20]. The resolved proteins were electroblotted onto nylon membranes (Cat IPVH00010 from Immobilon-P, Millipore, Ontario, CA). The unbound sites on the membranes were blocked with *casein (Vector Laboratories Catalog No SP-5020, concentration 1:10).* The active Caspase-3 protein bands were detected by incubating membranes with the active Caspase-3 specific mouse monoclonal antibody (mAb) from MBL (1:1000 dilution; catalogue # PM014). The secondary antibody was the Vectastain biotinylated anti-mouse IgG from Vector Laboratories. For IL-18BP, specific antibodies from R&D (catalogue #AF 119) were used. The secondary antibody used was biotinylated anti-goat IgG (H+L) from Vector Laboratories (Cat BA-5000). Anti human Myosin Light Chain 2 rabbit polyclonal antibody from Cell Signaling Technology (1:1000 dilution; catalogue # 3672). Phospho-Myosin Light Chain 2 (Ser19)-specific antibody was mouse mAb

from Cell Signaling Technology (1:1000 dilution; catalogue # 3675). Anti-Stat5 polyclonal antibody was from Cell Signaling Technology (1:1000 dilutions; catalogue # 9363). Phospho - Stat5-specific polyclonal antibody from Cell Signaling Technology (1:1000 dilution; catalogue # 9351). The IL-18 and IL-18BP antibodies that used for Western blot were also used in Immunofluorescence staining.

Immunofluorescence microscopy

Caco2 and HT-29 cells were seeded on round glass coverslips in six-well plates for four days. On the fourth day, the cells (confluence 60-80%) were treated with 10ng/ml IL-18 and Tat protein 100ng/ml for 24 hours and were used for Immunofluorescence staining. The cells were fixed and permeabilized by incubation with Methanol 50% and PFA 4% for 10 min on ice. After washing the coverslips three times by PBS containing 2% FBS for 5 minutes, the cells were incubated with 10% rabbit serum and 10% FBS for 1 hour on ice. The following antibodies were used in 4˚C over night as first antibodies: polyclonal rabbit anti-β-Catenin from Cell Signaling Technology (#9562), rabbit polyclonal anti human Occludin from Invitrogen (cat#40-4700). Anti-human Claudin-2 antibody (A-24) was rabbit polyclonal IgG from Santa Cruz (cat#133464). After 24 hours' incubation with the first antibody for at 4˚C, the cells were washed three times PBS. FITC-conjugated anti-rabbit F(ab') from eBioscience (Ref 11-4839-81) was used as the secondary antibody.. The secondary Ab was incubated with cells for 1h on ice. Actin filaments were stained by Tetramethylrhodamine B isothiocyanate (TRITC)-labelled Phalloidin from Sigma Aldrich (Catalog Number P1951) by incubation with cells for one hour on ice. Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) (at 1:5000 dilution) from Biolegend (cat#422801).

Virus preparation and treatment

Infectious proviral DNA for a T-tropic viral strain NL4.3 and a dual tropic viral strain 86.9 was amplified from their respective plasmids (pNL4.3 and p89.6, respectively) using a commercial kit (Midiprep kit, Qiagen, Ontario, CA). Viral preparations were made by transfecting HEK293-T with 1.00 ug plasmid DNA or without plasmid DNA for mock virus preparation. The transfected cells were incubated in the medium (RPMI) at 37°C in humidified 5% CO_2

atmosphere. After 24 hours, culture supernatants were collected, clarified by centrifuged at (14,000 *g*). The supernatants were titrated for p24 contents using a commercial ELISA kit from ABL (Rockville, USA). The supernatants were aliquoted and stored at -80°C. The supernatants from mock-transfected HEK293-T cells were used as mock viral preparations.

HT-29 cells were incubated with NL4.3 or p89.6 HIV-1 virus strains for 24 hours as described before [21]. After the incubation, the cells were washed 3 times with PBS and lysed for Western blot. The levels of IL-18 and IL-18BP in the supernatants were measured by ELISA after deactivating the virus by 1% Titron X-100 (Sigma-Aldrich) for one hour.

The Transendothelial Electrical Resistance (TEER) in Caco2 cells

Caco2 ($2x10^4$ cells) were seeded on a gelatin-coated 3-μm pore size Boyden chamber (from BD Biosciences). DMEM culture medium was changed every day. On the experiment day, Caco2 cells were treated with 10 ng/ml of IL-18 and⁄or with 100 ng/ml of HIVs' recombinant Tat protein (from ProSpec). For each treatment, three replicates were used. TEER was measured over 24 hours in treated and vehicle-treated monolayers. It was measured by the Electric Cell-substrate Impedance Sensing ECIS Zθ instrument and 8W10E+ electrode arrays were used. It was expressed as Ohm (resistance) X cm^2 (surface area of monolayer) as previously described [22].

Measurement of intestinal permeability by using Lucifer yellow in HT-29 cells

HT-29 cells were seeded on 24 wells plate (Boyden chambers inserts from BD Biosciences (353096) The 24 plates that are adapted to the test are from the same company cat# 353504). When the monolayers reached 90% confluence, recombinant human (r) IL-18 (10 ng/ml), pre-neutralized rIL-18 (10 ng/ml), Tat (100 ng/mL), pre-neutralized Tat (100 ng/mL) and IL-1β (10 ng/ml) were added to the chambers in apical or basal positions. After 16 h of the treatment, cells were washed 3 times with PBS containing 2% FBS. 250 ul of PBS were added to the lower chambers, and 100 ul of PBS containing 100 nmols of fluorescence tracer, Lucifer Yellow CH dilithium salt (Yellow Lucifer; 0.45 kD; Sigma-Aldrich), was added to the upper chambers. After 30 minutes upper chambers were removed and PBS were collected from the lower chambers and transferred to ELISA 96 wells plate to measure fluorescence intensity at 428 nm excitation and 530 nm emission wavelengths. The permeability of the monolayer was measured

by the following formula: $P_c = (V/(A \times C_i) \times (C_f / T)$, where P_c = Permeability measurement), V = volume of PBS of basal chamber inml, A = area of membrane insert in cm^2, C_i = concentration of LY added in μM, C_f = final concentration of LY in μM), and T = Time of essay in seconds. TEER was expressed as Ohm (Ω) (resistance) × cm^2 (surface area of monolayer)

Apoptosis Assay

The percentages of apoptotic cells were measured by Annexin V-FITC Apoptosis Detection Kit eBioscinece (REF# BMS500FI/300). Briefly, HT29 cells were collected by using the Versene Solution from Life Technology. Cells were stained by Annexin (V-FITC) for 15 minutes, washed and stained with propidium iodide (PE) and analyzed by flow cytometry.

Measurement of LPS, IL-18 and IL-18BP

Levels of lipopolysaccharide (LPS), IL-18 and IL-18BP in different samples were measured by using specific enzyme-linked immunosorbent assay (ELISA) kits obtained from MyBioSource (Catalog # MBS702450), eBioscience (REF# BMS267INST), and R&D Systems (catalog #DY119X), respectively. The detection limits of the kits were: 6.25 pg/ml -400 pg/ml for LPS, 93.8 - 6,000 pg/ml for IL-18BP, and 78-1000 pg/ml for IL-18.

Study Participants

Serum samples from three different categories of HIV-infected individuals (15 HIV-infected and anti-retroviral therapy (ART)-naïve patients, 15 HIV-infected and ART treated patients, 9 elite controllers (HIV-infected individuals who are able to spontaneously control HIV replication, with maintain HIV RNA levels below 50 copies/mL in the absence of therapy) [23] and 17 HIV seronegative healthy donors were used in this study. The study was approved by the institutional ethics committee. The samples were obtained from all the donors after their written informed consent, Clinical data for the HIV-infected individuals is given in Table 1.

Reagents

The reagents used in the study were: recombinant Human IL-18 (rh IL-18) from MBL Cat #BOO1-5, Serum free medium from Life Technologies, recombinant HIV-1 viral protein Tat from ProSpec Protein-Specialists, monoclonal IL-18 neutralizing antibody from MBL

(Cat#D047-3), monoclonal HIV Tat neutralizing antibody [AC11.AE12] from Abcam (Catalog # ab24778), Lipopolysaccharide (LPS) from Sigma-Aldrich (#L4005), ROCK inhibitor GSK 269962 from Santa Cruz (# sc-363279).

Statistical analysis

Group means were compared using ANOVA followed by Tukey's test. Pearson correlation coefficients between the parameters were determined. The software Graphad PRISM5 (San Diego, CA) was used for these analyses. Significance was deemed when p values were ≤ 0.05.

RESULTS

Effects of HIV infections and the viral protein Tat on IL-18 and IL-18BP production from HT-29 intestinal cells

The IEC line HT-29 is susceptible to HIV infection and produces both IL-18 and its antagonist [24, 25]. Therefore, we used these cells to investigate the potential effects of the infection and the viral transactivator Tat on the production of these two soluble mediators. The cells were infected and mock-infected with the CXCR4-tropic HIV strain (NL4.3), a dual tropic HIV strain (89.6), incubated with recombinant Tat or the vehicle. After 24 hours, the cells and the culture supernatants were collected. We detected precursor IL-18 (pIL-18), mature IL-18 (mIL-18) and IL-18BP in the cell lysates by Western blots using specific antibodies. As shown in Figure 1, the infection and Tat each caused increases in the levels of mIL18 but not in those of pIL-18. However, the infection and the treatment caused a decrease in the levels of IL-18BP in the cells as determined by Western blots (Figure 1A-C). A slight decrease in pIL-18 was observed only in NL4.3 that caused a more pronounced increases in mIL-18 probably due to more processing of the precursor form into its mature form. An increase in mIL-18 suggested increased processing of the pIL-18. As caspase-1 is mainly responsible for this processing, we sought to determine the effect of a caspase-1 inhibitor on the HIV-induced increase in mIL-18. As shown in Figure 2, the simultaneous treatment of the virus-infected cells with a cell permeable caspase-1 inhibitor prevented increase in mIL-18 inside cells but had no effect on IL-18BP levels.

Next we determined the effects of HIV infection and Tat treatment on the secretion of mIL-18 and IL-18BP. We measured their concentrations in the culture supernatants of the virus-infected and Tat-treated HT-29 cells. As shown in Figure 3A, the viral infections and the Tat treatment all increased mIL-18 concentrations in the culture supernatants significantly ($p>0.01$). On the other hand, these treatments decreased the concentrations of IL-18BP in the culture supernatants (Figure 3B). As expected, in this experiment, treatment of Tat with Tat-specific polyclonal antibodies abrogated the Tat-exerted effects on the secretion of mIL-18 indicating that the effect is specific to Tat, and not due to any contaminants present in this preparation.

IL-18 induces death in cells dependent upon dose and time

We and others have shown that IL-18 can induce death in certain cell types [20, 26], therefore we sought to determine the effects of recombinant human IL-18 on these IEC. The cytokine induced morphological changes in HT-29 cells grown in monolayers. The cells became

progressively rounded, detached and underwent death. Figure 4A shows cell death on day 5 after treatment of the HT-29 monolayers with IL-18 (100 ng per ml). As expected, prior incubation of the cytokine with an IL-18 neutralizing monoclonal antibody abrogated the death inducing effects of the cytokine. These data suggest that the effects of the cytokine were specific and were not due to any contaminant present in its preparation. In order to determine the mode of cell death, the cells were treated with the cytokine, and analyzed for their staining with FITC-conjugated Annexin V and PI at different time points. The cells became positive for Annexin V (a marker of early apoptosis) 6 hours after the treatment. Thereafter at 12 hours, they became positive for Annexin V and PI (advanced apoptosis), and finally at 24 hours they became positive for PI only (late apoptosis with loss of membrane integrity; Figure 4B). This pattern of temporal expression of the two stains is typical of apoptosis. We measured cell death (PI positive cells) at different doses of the cytokine. As shown in Figure 4C, and 4D, the cytokine induces cell death in a dose and time dependent manner. Remarkably, a subset of the cells remained resistant to death.

IL-18-induced cell death could be inhibited by caspase-1 and caspase-3 inhibitors

Since capase-1 and capase-3 can induce different types of cell deaths (pyroptosis and apoptosis, respectively), we sought to determine the caspase involved in the IL-18-induced cell death. For this purpose, the cells were incubated with the cytokine (50 ng per ml) with and without the presence of cell permeable inhibitors specific for one or the other caspase or together (50 μM). As shown in the Figure 5A, each of the caspase inhibitors partially inhibited death in HT-29 cells. Together, the two inhibitors had no added effect. These results suggest that both caspase-1 and caspase-3 cause same extent of cell death in this cell line.

IL-18 and LPS both activate caspase-1 and caspase-3 in HT-29 cells.

As the previous experiment suggested the involvement of caspase-1 and caspase-3 in the cytokine-induced death of HT-29 cells, we sought to determine whether the cytokine activates these caspases directly. For this purposes, the cells were treated with IL-18 (10 ng per ml) or LPS (10 ng per ml). LPS is known to activate both caspases and was used as a positive control [27]. The activation of the caspases was determined by Western blots using antibodies specific

for activated caspase-1 and activated caspase-3. As shown in Figure 6, LPS and IL-18 each activated the two caspases in HT29 cells.

IL-18 causes disruption and redistribution of β-catenin in HT-29 monolayers.

Since β-catenin plays an essential role in maintaining the integrity of adherens junctions by linking E-cadherin with the cytoskeleton in epithelial cell monolayers [28], we sought to determine the effects of IL-18 on the distribution of β-catenin in the cell monolayers. We used Tat as a positive control, as it is known to disrupt these junctions [29]. As shown in Figure 7, IL-18 causes an increased expression but abnormal distribution of this molecule in the epithelial cell monolayers. In contrast, Tat significantly reduces its expression. When added together, the effects of IL-18 seem to predominate over those of Tat.

IL-18 adversely affects expression of Tight Junction proteins in intestinal epithelial cell monolayers.

Tight junctions are the main structures that regulate paracellular passage of biomolecules across epithelial cell monolayers [13]. In this regard, occludin and claudin-2 are important proteins in these junctions. Therefore, we sought to determine the impact of IL-18 on these proteins in the IEC monolayers. As shown in Figure 8B, the cytokine reduced expression of occludin in the HT29 cell monolayers. In this respect, Tat behaves similar to the cytokine. As shown in Figure 8A, the cytokine treatment (10 ng per ml for 24 hours) markedly reduced expression of claudin-2 in Caco2 cell monolayers. Similar results were obtained when the expression of the proteins was determined by Western blots (Figure 8C). In this respect, the effect of Tat was less pronounced.

IL18 decreases the expression of F-actin and disrupts its normal distribution in the cell cytoplasm.

F- (or filamentous) actin is important for maintaining cell shape and motility in epithelial cells as well as for regulating intestinal permeability [30]. Therefore, we investigated the impact of IL-18 treatment (with and without Tat). As shown in Figure 9, both IL-18 and Tat caused

reductions and others abnormalities in the expression of this cytoskeletal protein in Caco2 cell type. Same effect was observed with HT-29 cells (data not shown).

IL-18 increases permeability in the intestinal epithelial cell monolayers.

As IL-18 causes cell death and several changes in cell shape and in the expression of cytoskeletal and Tight Junction proteins, the cytokine may adversely affect paracellular permeability in the intestinal epithelial cell monolayers. Therefore, we sought to investigate the impact of this cytokine on paracellular permeability in Caco2 cell monolayers by measuring trans-epithelial electrical resistance (TEER). As shown in Figure 10, the cytokine induced a decrease in the TEER that began at hour 6 post-exposure. The decrease persisted until 24 hour post-exposure. Tat and IL-18 had similar effects on the TEER of the cell monolayer after 24 hours of treatment.

IL-18 affects permeability when applied from apical but not from the basolateral surface of the intestinal epithelial cells.

It has been shown that cytokines's effects on paracellular permeability in epithelial cell monolayers may depend upon whether they are applied to the apical or to the basolateral surface [31, 32]. Therefore, we sought to determine whether application of IL-18 to the apical or basolateral surface of the HT-29 monolayer affected its ability to increase the permeability. To this end, we grew the cells in Transwells. When confluent, we added the cytokine either to the apical surface or the bottom well. After 24 hours' exposure, we added the Lucifer Yellow tracer to the top well, and measured its concentrations in the bottom wells 30 minutes later. As shown in Figure 11A, IL-18 increased permeability when applied to the apical surface. Neutralization of the cytokine with the cytokine neutralizing antibodies abrogated this effect. The cytokine, however, had no effect on the monolayer permeability when it was applied from the basolateral surface (Figure 11B). Contrary to this, IL-1β exerted its effect on permeability from the basolateral, but not from the apical, application. Tat increased the monolayer permeability from both the surfaces.

IL-18 increases expression of MLCK and induces phosphorylation of MLC via ROCK.

Some other cytokines (e.g., TNF-α and IL-1β) have been shown to disrupt intestinal integrity by increasing expression of MLCK and inducing phosphorylation of MLC by a pathway that involves ROCK [33]. Therefore, we were interested in determining whether IL-18 also follows the same pathway for disrupting integrity of HT-29 monolayers. For this purpose, we treated the cell monolayers with IL-18 (10 ng per ml) and or Tat (100 ng per ml), and determined the expression of MLCK and pMLC at different time points using Western blots. As shown in Figure 12A, the cytokine increased expression of MLCK, which could be detected 30 minutes after treatment of the monolayers with the cytokine. Interestingly, at the 10 and 60 minutes post-treatment, the expression was decreased relative to untreated monolayers. The effect of Tat was similar to that of IL-18. Additive effect was seen for the two treatments at the 10 minutes' time point (Figure 12A). Consistent with the IL-18-induced increased expression of MLCK, the cytokine induced increased phosphorylation of MLC at all the tested time points (10, 30 and 60 minutes post-treatment; Figure 12B. To our surprise, Tat decreased pMLC in the monolayer cells at 30 minutes after the treatment despite increasing expression of MLCK at this time point (Figure 12A and 12B). Both IL-18 and Tat tended to reduce pSTAT-5 in the cell monolayers when they were examined 30 minutes after the treatments (Figure 12C). In order to determine whether ROCK was involved in the phosphorylation of MLC, we determined the effects of a cell permeable inhibitor of ROCK. The inhibitor reduced the expression of pMLC in IL-18 treated HT29 monolayers both at 10 and 30 minutes after their treatment with the cytokine (Figure 12D). Collectively, these data suggest that IL-18 uses the same signaling pathway for disrupting integrity of intestinal epithelial cell monolayers that are used by TNF-α and IL-1β [34-36].

Serum concentrations of IL-18 and LPS correlate with each other in HIV-infected individuals and healthy controls.

As we determined that IL-18 was a determinant of intestinal permeability, and microbial translocation has been shown to be a major factor in aberrant immune activation and AIDS pathogenesis [37, 38], we sought to determine whether serum concentrations of IL-18 correlated with those of LPS in the serum. To investigate this, we measured IL-18 in the sera of HIV-infected patients belonging to different categories i.e., treatment-naïve HIV-infected, HAART-treated HIV-infected, elite controllers and HIV-seronegative healthy control subjects (Figure 13

A, B). The treatment naïve HIV-infected patients had significantly higher concentrations of IL-18 and LPS compared with the control subjects ($p<0.05$). In these respects, the elite controllers were not different from healthy individuals. More importantly, in all the donor groups including healthy control subjects, significant correlations were observed between IL-18 concentrations and LPS levels (Figure 13 C-F). These data strongly suggest that IL-18 is as a major factor in increasing intestinal permeability and causing microbial translocation in HIV-infected individuals as well as in healthy subjects.

DISCUSSION

We show here for the first time that HIV increases expression of the precursor and mature IL-18 in human intestinal epithelial cell line. However, the virus decreases the expression of IL-18BP, a naturally occurring antagonist of IL-18. We also found that viral infection increases concentrations of mature IL-18 but reduces those of IL-18BP in the culture media of these cells. Previous studies from our [4] and other laboratories [9, 39] have shown that concentrations of

IL-18 are increased in the circulation of HIV-infected individuals but those of its antagonist are either decreased or are not correspondingly increased. This imbalance in the production of IL-18 and IL-18BP results in increased amounts of free, biologically active IL-18 in the circulation of HIV-infected individuals. Treatment of HIV-infected patients with antiretroviral drugs reduces these levels but they tend remain higher than their normal levels seen in HIV-seronegative healthy individuals.

IL-18 is produced from a wide variety of human cells including monocytes, macrophages, dendritic cells, intestinal epithelial cells as well as from the adrenal cortex [40, 41]. Contrary to the cytokine, production of its antagonist in the body is more ubiquitous. In fact it is produced as a negative feedback in response to a rise in the levels of IL-18, either locally or systemically. Most of IL-18 in the circulation is bound with IL-18BP and is inactive. An increase in its production also results in increased production of IL-18BP. IL-18 does so by inducing IFN-γ from NK, T and other cell types in the body. Production of IFN-γ is essential for transcriptional activation of the gene for IL-18BP, as IFN-γ KO mice have no detectable IL-18BP in their circulation [42]. Previously, we have shown that both T- and M-tropic viruses increase expression of IL-18 and decrease that of IL-18BP from human monocyte-derived macrophages [4]. We also showed that replication of the virus was required for these effects. Our present results suggest that the virus exerts similar effects on intestinal epithelial cells. It seems that the virus induces imbalance in IL-18 and IL-18BP from more than one cell type. In this connection, platelets may also contribute to this imbalance. It was shown previously that IL-18 concentrations in the circulation of HIV-infected individuals correlate with the degree of platelets activation [2]. In this connection, we have recently found that human platelets can produce both IL-18 and IL-18BP, and HIV-1 interacts with these cells and induces expression of IL-18 but reduces that of IL-18BP (manuscript included in the thesis). Furthermore, the adrenal cortex may also contribute towards this imbalance. HIV-infected individuals are likely to experience increased psychosocial stress, which is known to activate the hypothalamus-pituitary-adrenal (HPA) axis and results in increased outputs from both adrenal medulla and cortex [43].

The imbalance in the production of IL-18 and its antagonist inevitably results in increased biological activities of IL-18, which may have tissue destructive effects. The cytokine has been shown to induce death in several human cell types, including human cardiac and microvascular

endothelial cells [6, 44]. We have previously shown that recombinant human IL-18 induces FasL expression on human NK cells. The IL-18-treated NK cells exert fratricidal effects via Fas-FasL interactions [20]. As HIV replicates intensively in gut-associated lymphoid tissues, and the virus induces this cytokine from the intestinal epithelial cells, we sought to determine potential effects on these cell monolayers. The cytokine decreased trans-epithelial electrical resistance (TEER) in fully confluent intestinal epithelial cell monolayers. The IEC line we used in these assays, Caco-2, has been extensively used for this purpose [45]. It is noteworthy that TEER is an indirect measure of the paracellular permeability of the cells grown in monolayers [46, 47]. These results suggest that IL-18 increases intercellular permeability in these cell monolayers. We also measured permeability of the fully confluent IEC monolayers by using a fluorescent tracer (Lucifer Yellow). Being 0.45 *kDa* in size, the tracer crosses the intestinal cell monolayer only if the tight junctions' permeability is increased. Interestingly, IL-18 increased permeability of the HT-29 monolayers when it was applied to the apical surface of the monolayer but not when it was applied to its basolateral surface. On the contrary, IL-1β, which is the prototype member of the IL-1 family, increases the permeability from basolateral surfaces but not apical surfaces. Our results on the effects of IL-1β on the intestinal permeability are in agreement with earlier studies [48]. These differential effects of IL-18 and IL-1β on the two surfaces of the IEC monolayers may be due to differential expression of their receptors on these surfaces. The receptors for IL-1β appear to be expressed on basolateral surfaces of the IEC, whereas those of IL-18 may be restricted to its apical surface only. However, IL-18 shows faster and continuous decrease in permeabilty when measured by TEER than IL-1β after cells treatment. IL-1β induce transit increase in TEER flowed by continuous decrease. We suggest this difference in induced permeability related to apoptosis induced by IL-18 but not IL-1β. Further studies are required to test these possibilities.

We report here for the first time IL-18 increases intestinal permeability. The cytokine induces increased expression of MLCK and induces phosphorylation of MLC. The phosphorylation of MLC is essential to cause contraction of the actomyosin cytoskeleton and an increase in the tight junction permeability [49]. We also noted significant changes in the expression and distribution of the tight junction and junctions adherens proteins. We observed a decreased expression of occludin and claudins. The expression of claudin-2 was also disrupted. The latter protein is known to be porous, and its increased/disrupted expression correlates with increased

permeability of the tight junctions [50]. Concerning intestinal permeability, IL-18 exerts effects similar to those of other pro-inflammatory cytokines like IL-1β, TNF-α, IL-6 and IFN-γ [51-53]. However, the relative role of each of these cytokines in HIV-induced increased intestinal permeability and enteropathy remains unknown. Increased intestinal permeability results in increased translocation of bacterial products and small fragments into body tissues and general circulation [54]. Microbial products like LPS cause a generalized activation of the immune system, which is invariably observed in HIV-infected individuals [16]. We also investigated concentrations of LPS and IL-18 in the circulation of HIV-infected and HIV-seronegative healthy individuals. Interestingly, we found significant positive correlations between these two parameters in different HIV-infected individuals as well as in healthy donors. We are not aware of any other pro-inflammatory cytokine which has been reported to correlate with the concentrations of the circulating LPS. These results suggest that IL-18 plays a fundamental role in regulating paracellular permeability in IEC monolayers.

We observed that addition of IL-18 to the intestinal epithelial cell monolayers caused significant changes in their morphology. The cells became rounded, died and started floating in the culture medium. We observed that the cells underwent apoptosis, as they first became positive for FITC-Annexin V (a marker for early apoptosis) and then for a vital dye, PI (a marker for late apoptosis when cells lose integrity of their membranes). The cytokine also induced activation of caspase-1 and caspase-3 in the intestinal cells. Furthermore we observed that the cytokine increased apoptosis in the IEC monolayers. It is noteworthy that a decreased turnover of enterocytes occurs in HIV-induced enteropathy [55]. Consequently, the villi become atrophied [56]. Our results suggest that virus-induced IL-18 is likely to play an important role in the enteropathy observed in HIV-infected individuals. As mentioned in the Results section, a subset of the IEC remains viable and resistant to apoptosis. While the exact reasons for this resistance remain unkonown. One possibility is that only a subset of the IEC differentiated and expressed receptors for the cytokine and became susceptible to the cytokine-induced death.

Recently, it was reported that incomplete reverse transcripts are sensed by an interferon-induced DNA sensing molecules (IFIT-16), which causes activation of caspase-1 and cell death [57]. Caspase-1 activation causes a unique type of death called pyroptosis, literally meaning death with fire [58]. This mode of cell death is accompanied by the release of pro-inflammatory cytokines including IL-1β and IL-18. It was reported that about 95% of CD4+ T cells express

defective HIV RT products and die from pyroptosis. It is not surprising that caspase-1 inhibitors prevents HIV-induced pyropotosis in CD4+ T cells [59]. It would be very interesting to investigate whether IL-18 released in the process plays a role in this type of cell death.

Increased concentrations of IL-18 are observed in the circulation of HIV-infected individuals. The use of antiretroviral therapy effectively suppresses viral replication below detection limits [60]. In this connection, it has been suggested that anti-retroviral drugs also contribute to keep the cytokine levels above their normal limits [39]. Increased levels of the cytokine induces chronic low-grade inflammation that puts HIV-infected individuals at risk for immunosenescence, aging, frailty, metabolic syndrome, cardiovascular diseases, dementia and HIV-associated lipodystrophy (HALS) characterized by the disappearance of sub-cutaneous fat ("empty cheek syndrome") and its accumulation at unusual anatomical sites like the back of neck ("buffalo hump"), etc [61-63]. It is noteworthy that IL-18 inhibits differentiation of pre-adipocytes into adipocytes and promotes lipolysis [64, 65]. Increased expression of the cytokine was observed at the sites of depletion of subcutaneous fat in HIV-infected individuals [66]. Increased concentrations of the cytokine are a risk factor for heart attack, type 2 diabetes and dementia [67, 68]. Today, HIV-infected individuals on antiretroviral therapy rarely die of AIDS, but suffer from chronic low-grade inflammation that leads to several clinical conditions not associated with AIDS. Increased concentrations of IL-18 play an essential role in inducing these conditions.

We used Tat in our studies along with IL-18 to determine its effects on intestinal epithelial cells. Tat has been shown to play a role in the pathogenesis of HIV-induced AIDS [69]. We found that Tat also exerts similar effects on the IEC monolayers. Furthermore, it had additive effects when used in combination with the cytokine. Thus, IL-18 and Tat seem to promote cell death in IEC and contribute towards enteropathy observed in HIV-infected individuals. Although, the human IEC lines have been extensively used by researchers as in vitro models for conducting research on intestinal permeability, they are transformed self-renewing cancer cells. The results obtained from them should be accepted with this caveat. Ideally, they should be verified in *in vitro* studies in primary intestinal epithelial cell cultures or in appropriate animal models.

In summary, our results show that HIV infection of IEC results in differential effects on the production of IL-18 and its antagonist. It also induces activation of caspase-1 resulting in

processing of precursor IL-18 and the release of mature IL-18 into the culture media. The cytokine increases intestinal permeability and translocation of microbial products into systemic circulation. It also activates caspase-1 and caspase-3, and promotes apoptosis of IECs. The cytokine is very likely to play an important role in HIV-induced enteropathy. It may be considered a potential target molecule for reducing HIV-induced pathology.

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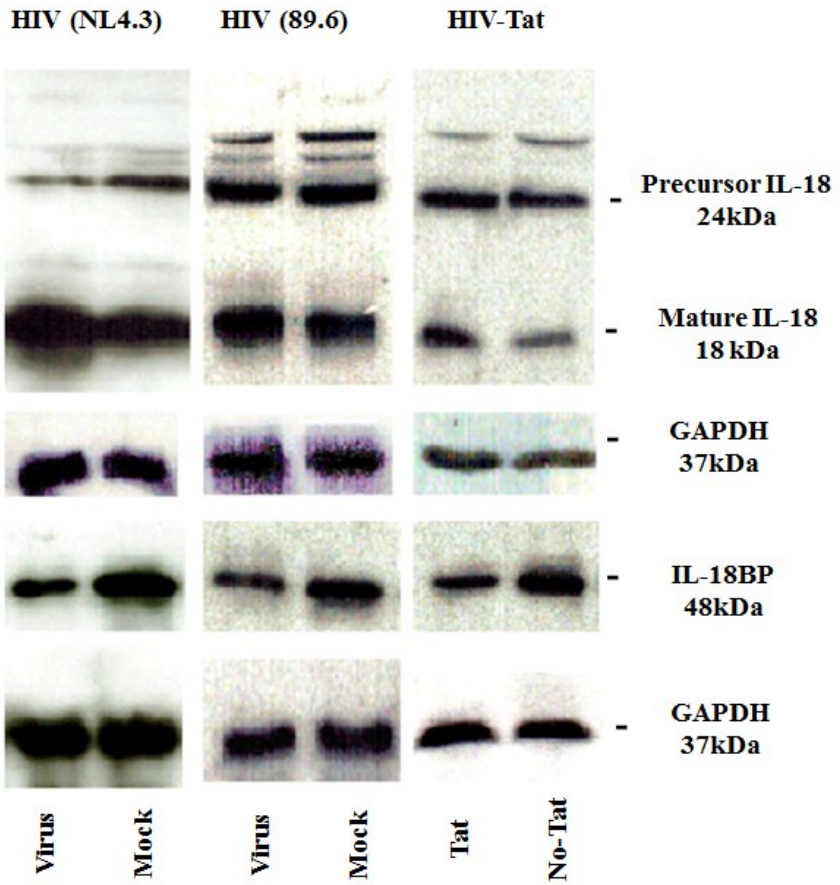

HIV (NL4.3)
HIV (89.6)
HIV-Tat
Precursor IL-18
24kDa
Mature IL-18
18 kDa
GAPDH
37kDa
IL-18BP
48kDa
GAPDH
37kDa
Virus
Mock
Virus
Mock
Tat
No-Tat

Figure 1

Figure 1. HIV increases production of IL-18 but decreases that of IL-18BP in IEC

HT-29 monolayers, containing about 1.00 million cells, were infected with 10^6 infectious units of NL4.3 or 89.6 HIV strains or treated with Tat (100 ng/ml). After 24 hours, IL-18 and IL-18BP were detected in cell lysates (25 ug of the lysate proteins) by Western blots. GAPDH was detected as control for protein loading. Note that the viral infections as well as the Tat treatment increase mature IL-18, decrease IL-BP with some effects on precursor IL-18.

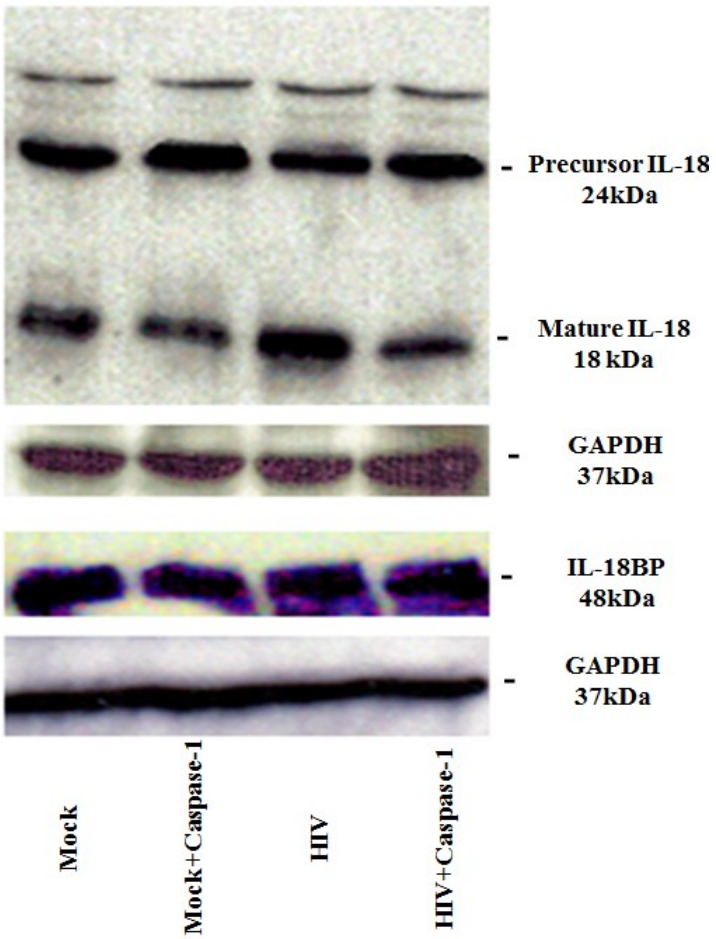
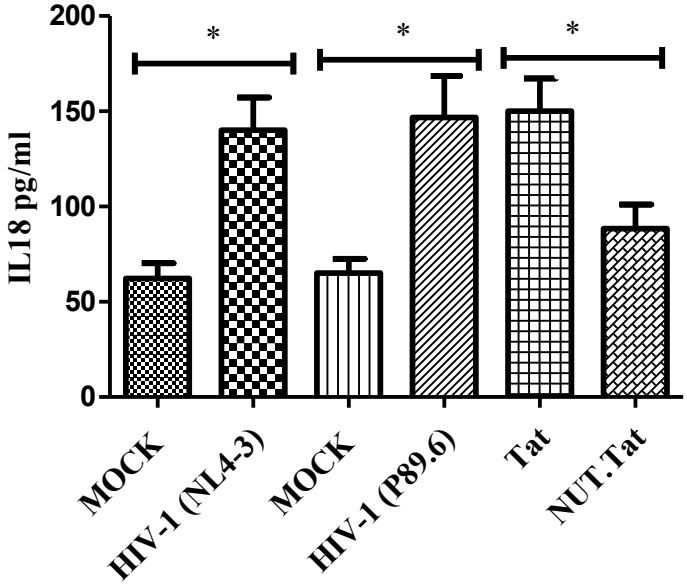

Precursor IL-18
24kDa
Mature IL-18
18 kDa
GAPDH
37kDa
IL-18BP
48kDa
GAPDH
37kDa
Mock
Mock+Caspase-1
HIV
HIV+Caspase-1

Figure 2

Figure 2. The effect of caspase-1 inhibitor on HIV-induced changes in IL-18 and IL-18BP in IEC.

HT-29 monolayers were infected for 24h with NL4.3. For every monolayer, one ml of the viral preparation containing 91 ng of p24 was used with or without prior 6 hours' treatment with caspase-1 inhibitor (Z-YVAD-FMK; 50 uM). The expression of the cytokine and its inhibitor was determined in cell lysates by Western blots.

A


200
150
100
50
0
IL18 pg/ml
*
*
*
MOCK
HIV-1 (NL4-3)
MOCK
HIV-1 (P89.6)
Tat
NUT.Tat

B

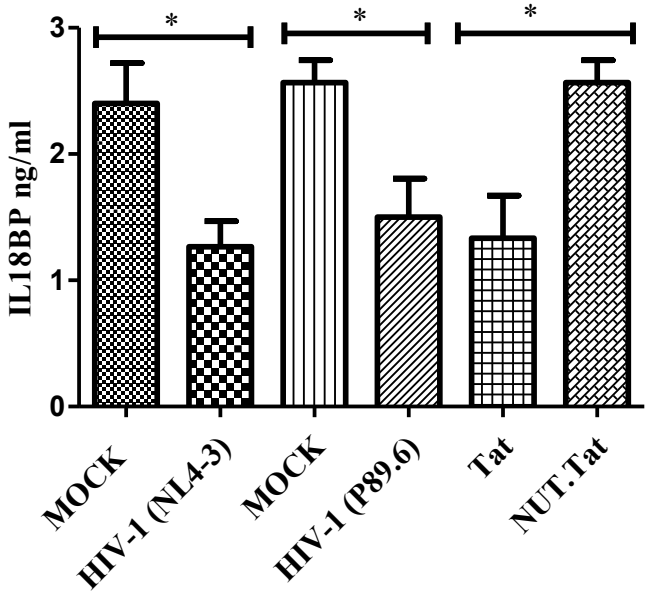

3
2
1
0
IL18BP ng/ml
*
*
*
MOCK
HIV-1 (NL4-3)
MOCK
HIV-1 (P89.6)
Tat
NUT.Tat

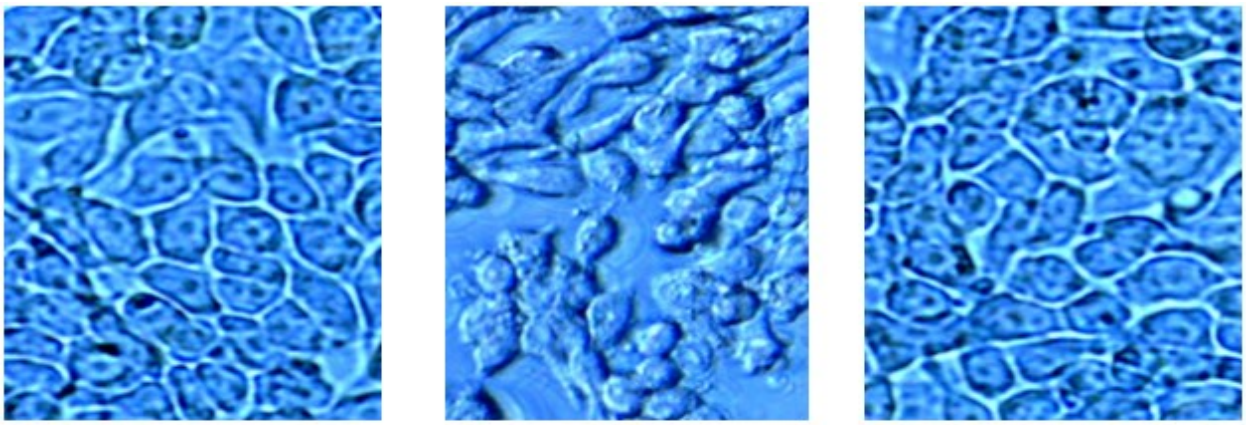
Figure 3

Figure 3. The effects of HIV and Tat on the secretion of IL-18 and IL-18BP from IEC.

After 24h of the viral infections and Tat treatment, supernatants were collected and cytokine levels were measured by ELISA. The panels A and B show concentrations (mean±SD) of IL-18 and IL-18BP, respectively, in the culture supernatants.

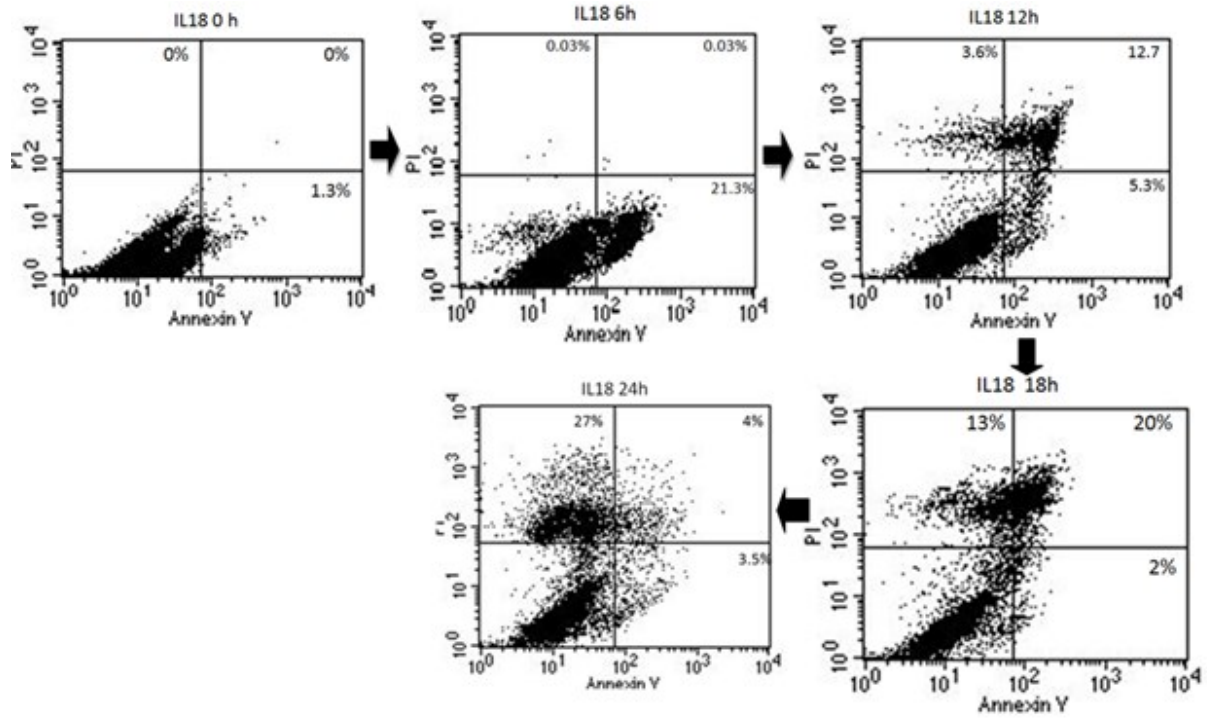
* indicates significant ($p<0.05$) difference. NUT.Tat means Tat protein neutralized by anti-Tat antibody.

A

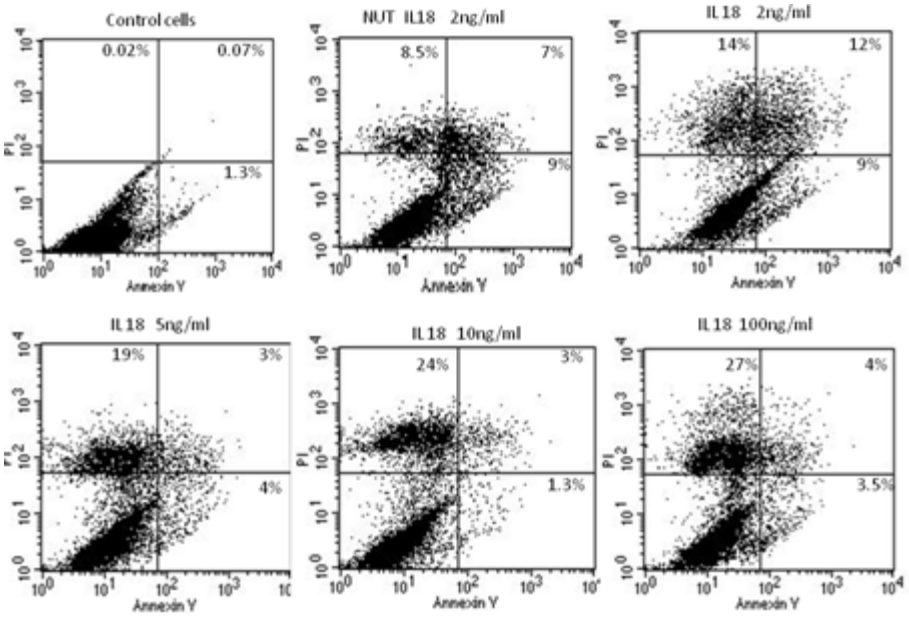


Vehicle IL-18 Neutralized IL-18

B



C


Control cells
0.02%
0.07%
1.3%
NUT IL18 2ng/ml
8.5%
7%
9%
IL18 2ng/ml
14%
12%
9%
IL18 5ng/ml
19%
3%
4%
IL18 10ng/ml
24%
3%
1.3%
IL18 100ng/ml
27%
4%
3.5%
PI
Annexin V

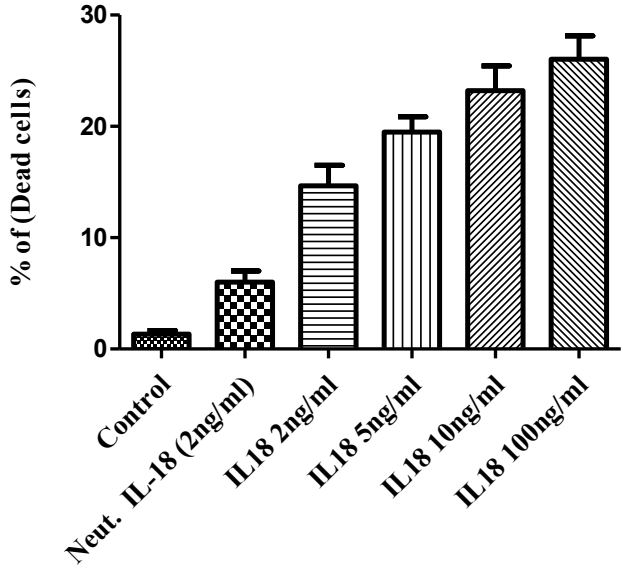

% of (Dead cells)
30
20
10
0
Control
Neut. IL-18 (2ng/ml)
IL18 2ng/ml
IL18 5ng/ml
IL18 10ng/ml
IL18 100ng/ml

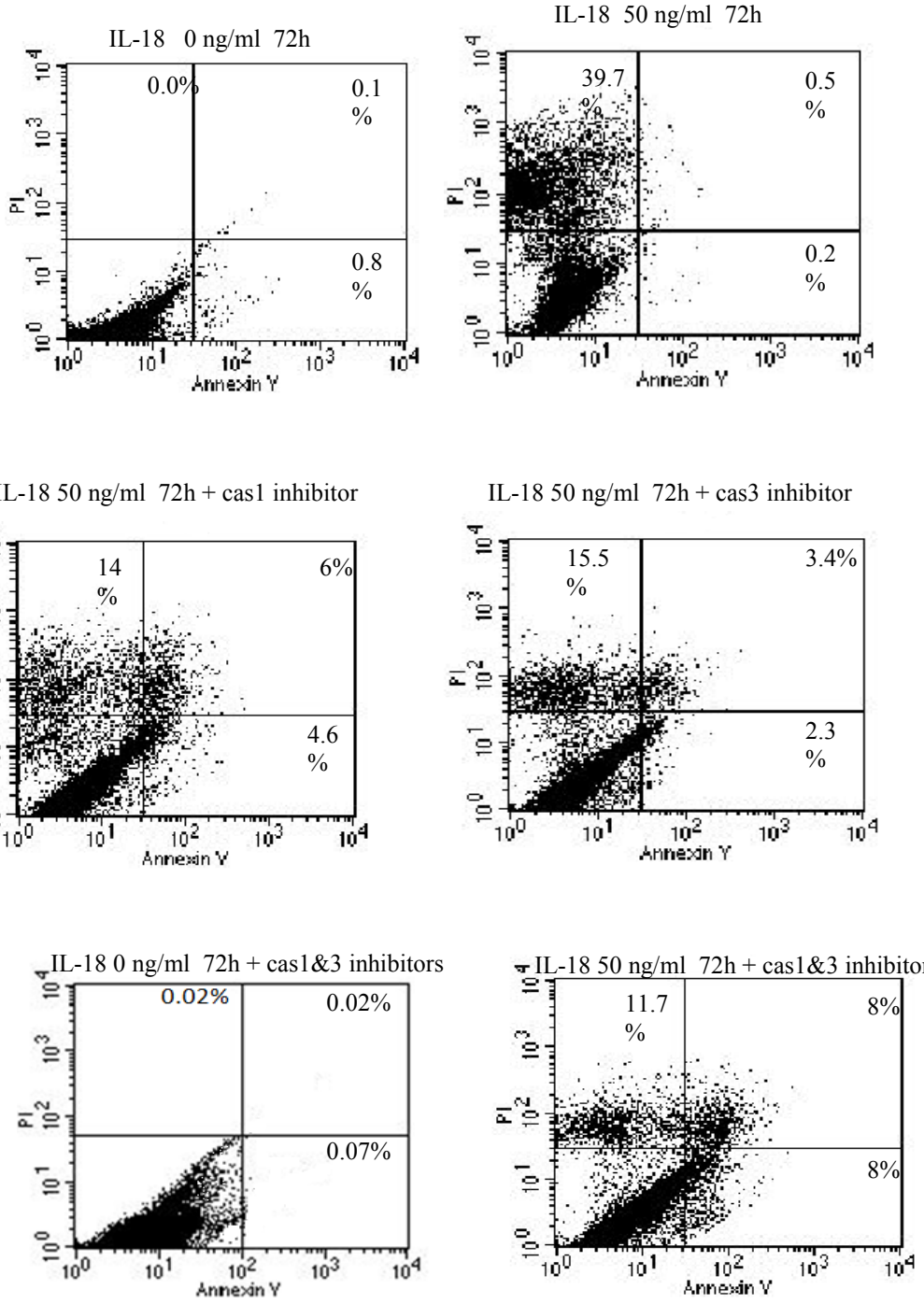
Figure 4

Figure 4. IL-18 induces death in HT-29 cells.

(A). Light microscope images of cells taken on day 5 after treatment with 100 ng per ml of IL-18. Note that prior neutralization of the cytokine with an IL-18 neutralizing monoclonal antibody (10 ug per ml) inhibited the deleterious effects of the cytokine. (B). Staining of the IL-18-treated cells with FITC-conjugated Annexin V and PI at different time points (from 0h to 24h). Note earlier staining of the cells with FITC-Annexin V followed by uptake of PI. The temporal pattern of the two stains is characteristic of apoptosis. (C). The cytokine induces death in a dose dependent manner. Each dose induced significantly more cell death compared with the untreated cells.

The neutralization of IL-18 inhibits death significantly ($p<0.05$).

A


IL-18 0 ng/ml 72h
0.0%
0.1 %
0.8 %
IL-18 50 ng/ml 72h
39.7 %
0.5 %
0.2 %
IL-18 50 ng/ml 72h + cas1 inhibitor
14 %
6%
4.6 %
IL-18 50 ng/ml 72h + cas3 inhibitor
15.5 %
3.4%
2.3 %
IL-18 0 ng/ml 72h + cas1&3 inhibitors
0.02%
0.02%
0.07%
IL-18 50 ng/ml 72h + cas1&3 inhibitors
11.7 %
8%
8%
PI
Annexin V

B

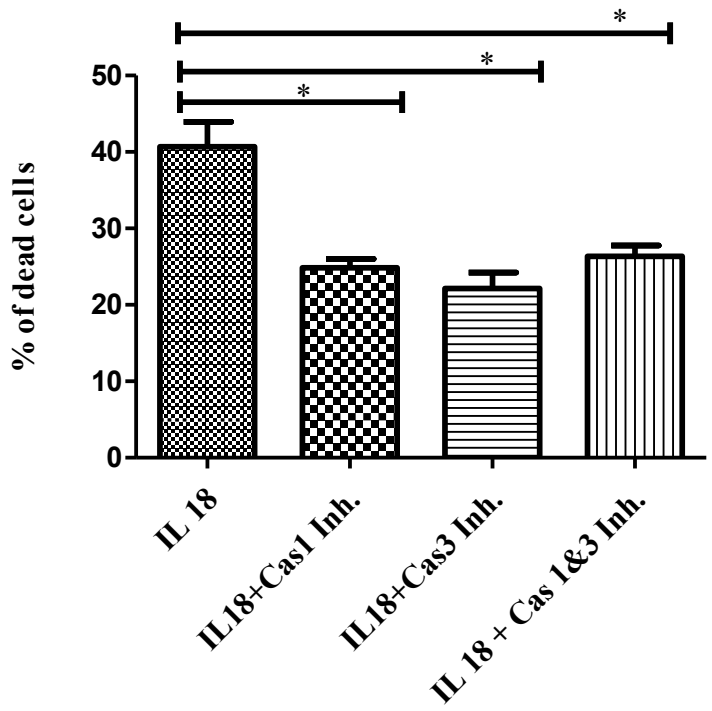

*
*
*
50
40
30
20
10
0
% of dead cells
IL 18
IL18+Cas1 Inh.
IL18+Cas3 Inh.
IL 18 + Cas 1&3 Inh.

Figure 5

Figure 5. IL-18-induced cell death is partially inhibited by inhibitors of caspase-1 and caspase-3.

HT29 cells were treated with IL-18 (50 ng /ml) for 72 hours. The cultures were treated with inhibitors for caspase-1 (Z-YVAD-FMK), caspase-3 (Z-DQMD-FMK) or both (50 uM each) for 4 hours. Then apoptotic cells were determined by staining with FITC-annexin V and PI. Panel A shows histograms of the cells stained for Annexin V and PI. Panel B summarizes the results from three independent experiments by showing mean %ages ± SD.
* indicates significant ($p>0.05$) difference.

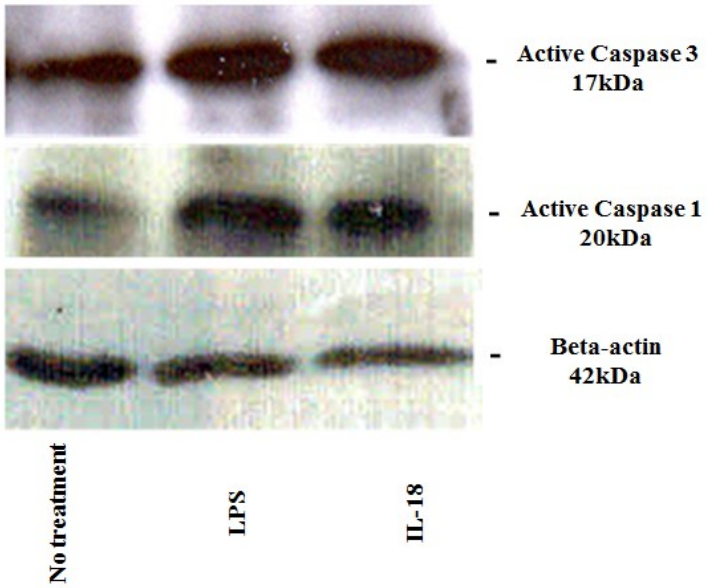

Active Caspase 3
17kDa
Active Caspase 1
20kDa
Beta-actin
42kDa
No treatment
LPS
IL-18

Figure 6

Figure 6. IL-18 and LPS activate caspase-1 and caspase-3 in HT-29 cells.

The cells were treated with IL-18 (10 ng/ml) or with LPS (10 ng/ml). After 4 hours, the cells were washed with PBS, lysed and the activation of the caspases was determined on Western blots by using antibodies specific to the activated forms of the caspases.

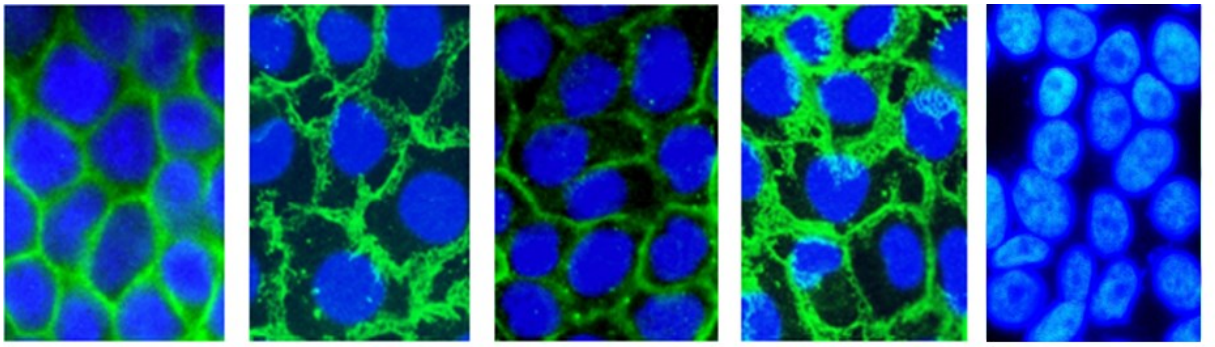
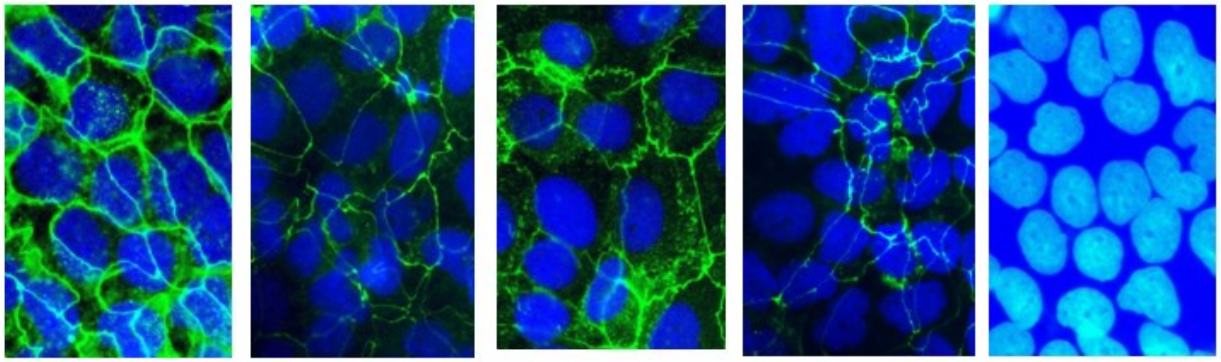


Figure 7

Figure 7. IL-18 disrupts and redistributes β-Catenin in Adherent Junctions in HT-29 monolayers.

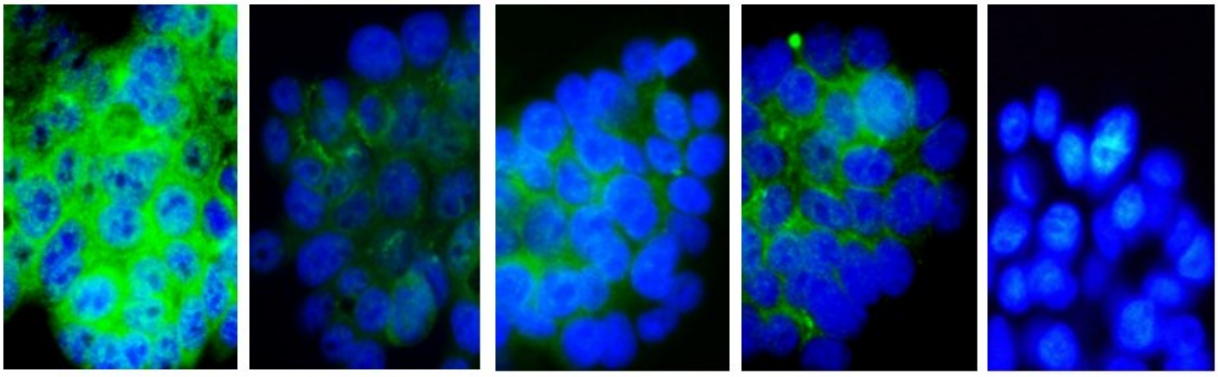
The HT-29 monolayers were treated with IL-18 (10 ng/ml), Tat (100 ng/ml) or both. After 24 hours, the monolayers were incubated with rabbit anti-human β-catenin or a control rabbit antibody (negative control). After washing, the cells were stained with FITC-conjugated goat anti-rabbit antibodies, counterstained with DAPI, washed and examined under a fluorescent microscope (Eclipse e-800 microscope, Nikon). The images were taken at 400X magnification. The Figure shows typical images. Note displacement and redistribution of the molecule around individual cells in the monolayers.

A



No IL-18 IL-18 Tat IL-18+Tat Negative control

B



No IL-18 IL-18 Tat IL-18+Tat Negative control

C

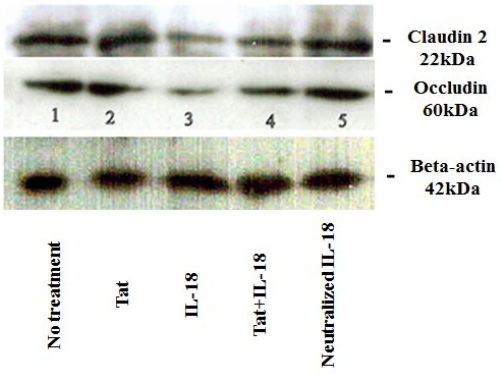
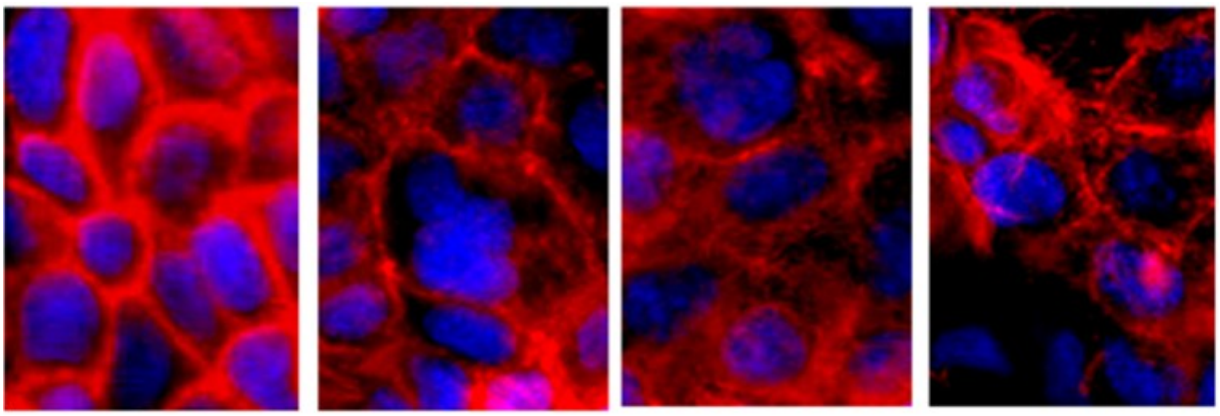


Figure 8

Figure 8. The effects of IL-18 on the expression of Tight Junction proteins in the intestinal epithelial cell monolayers.

The cell monolayers were treated with IL-18 (10 ng/ml) and/or with Tat (100 ng/ml) for 24 hours, after which the cell monolayers were washed with PBS and stained with either protein specific rabbit antibodies or control antibodies (negative control), washed and stained with FITC-conjugated goat anti-rabbit antibodies. The cells were also stained with DAPI and examined under a fluorescent microscope (Eclipse e-800 microscope, Nikon). The photomicrographs were taken under 400X magnification. **A**. Staining of Caco2 cells for claudin-2. **B.** Staining of HT-29 monolayers for occludin. **C.** Expression of claudin-2 and occludin in HT29 by Western blots.



No treatment **IL-18** **Tat** **IL-18+Tat**

Figure 9

Figure 9. Effects of IL-18 on the expression and distribution of F-actin.

The cells Caco2 monolayer was treated with IL-18 and/or with Tat for 24 hours. The cells were washed with PBS, permeabilized, stained with Phalloidin red and DAPI. The cells were examined under a fluorescent microscope (Eclipse e-800 microscope, Nikon) and photographed under 400X magnification. The cytokine and Tat decrease expression of F-actin and cause abnormalities in its distribution. Note less density of the staining on the cell periphery and its variable extension the cell interior in IL-18 and Tat-treated cells. The figure represents three time repeated experiments.

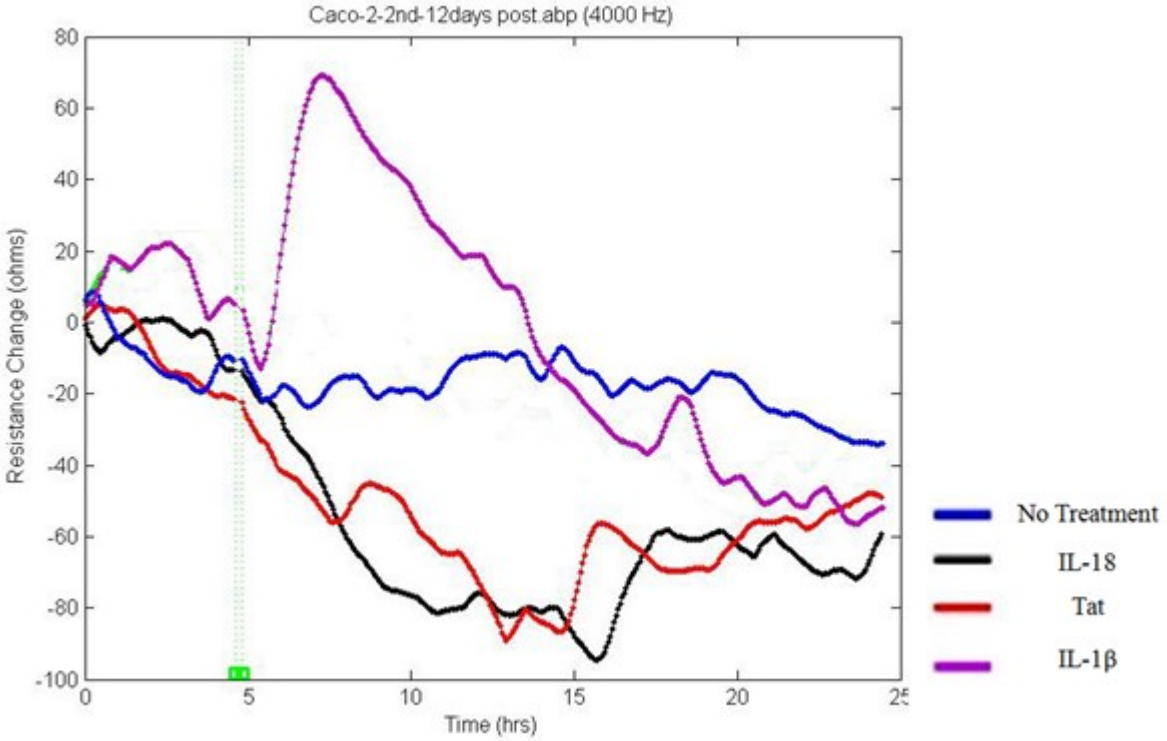
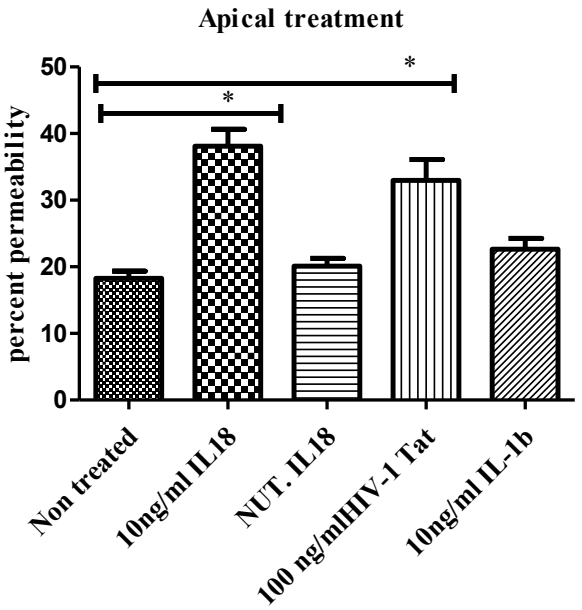

Caco-2-2nd-12days post abp (4000 Hz)
Resistance Change (ohms)
Time (hrs)
80
60
40
20
0
-20
-40
-60
-80
-100
0
5
10
15
20
No Treatment
IL-18
Tat
IL-1β

Figure 10

Figure 10. IL-18 decreases TEER in Caco2 monolayers.

The TEER was measured during 24 hours after addition of 10 ng/ml of IL-18, 10 ng/ml IL-1β or 100 ng/ml Tat to confluent cell monolayers. Note dramatic decrease of the TEER occurring at hour 6 post-exposure by both IL-18 and Tat. IL-1β causes a transient increase in the TEER followed by a decrease beginning at hour 14 post-exposure. TEER was measured by the Electric Cell-substrate Impedance Sensing ECIS Zθ instrument and 8W10E+ electrode arrays were used. The Figure shows changes in electrical resistance in ohms on X-axis and duration of treatment with the reagent on Y-axis. The figure represents three time repeted expermints.

A


Apical treatment
percent permeability
50
40
30
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10
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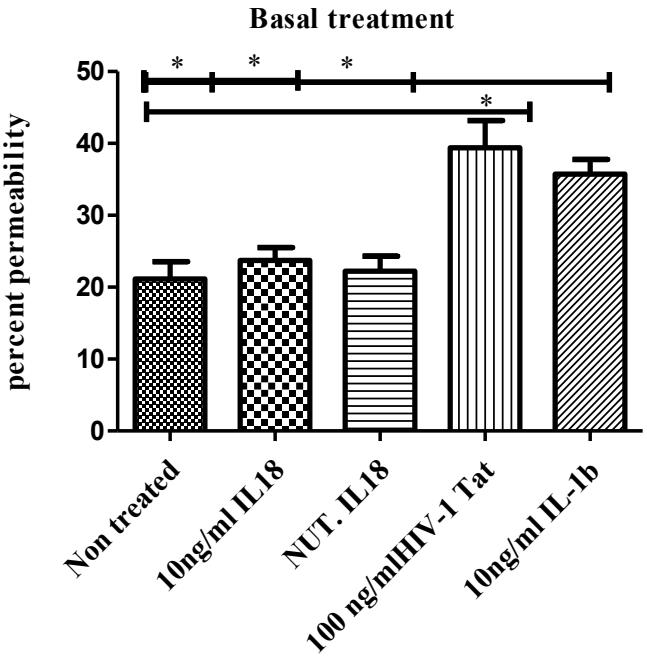
Non treated
10ng/ml IL18
NUT. IL18
100 ng/mlHIV-1 Tat
10ng/ml IL-1b

B


Basal treatment
percent permeability
50
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10
0
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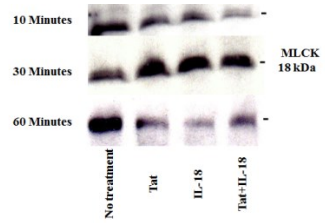
Non treated
10ng/ml IL18
NUT. IL18
100 ng/mlHIV-1 Tat
10ng/ml IL-1b

Figure 11

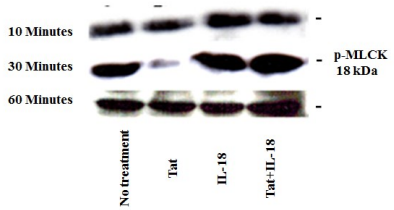
Figure 11. IL-18 increases paracellular permeability when applied to apical surface of the monolayers.

HT-29 cells were grown in monolayers in Transwell chambers. When confluent, IL-18 (10 ng /ml), IL-1β (10 ng/ml) or Tat (100 ng/ml) were added to the upper **(A)** or to the lower Transwell wells (**B**). In one well, we added IL-18 that was pre-neutralized with anti-IL-18 antibody (Neut IL-18). After 24 hours' exposure, Lucifer Yellow (0.45 kDa; 100 nM) was added to the top wells. After 30 minutes, the upper Transwell upper chambers (inserts) were removed, and the concentrations of the fluorescent tracer were measured in an ELISA reader. Percent permeability was determined by comparing the tracer in each of the lower wells with a well to which the tracer was added (100 nM). Bars and vertical lines indicate means ± SD. * indicates that $P<0.05$.

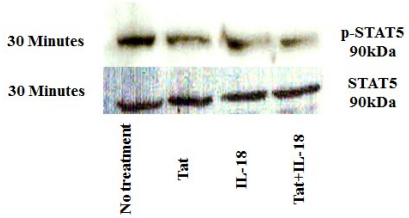
A



B



C



D

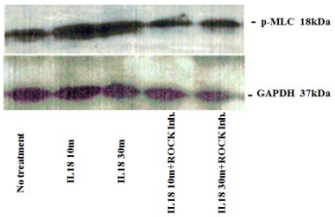
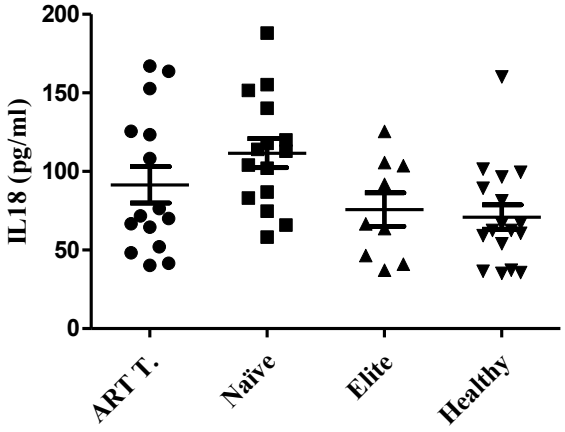


Figure 12

Figure 12. IL-18 increases expression of MLCK and pMLC via ROCK.

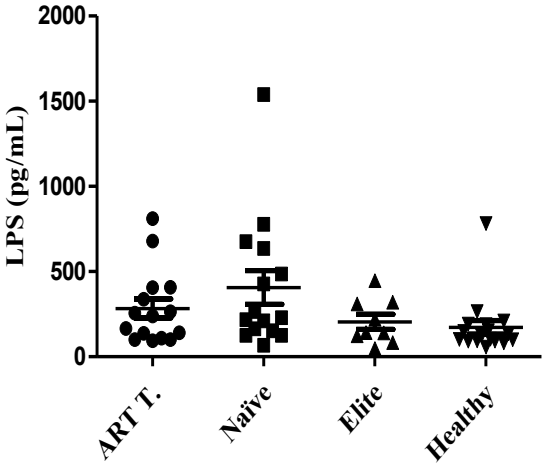
HT29 cell monolayers were treated with IL-18 (10 ng/ml) and/or Tat (100 ng/ml). At the indicated time points, the cells were lysed. About 25 µg of the lysate proteins were resolved on SDS-PAGE and the Western blots were processed for the revealing of MLCK (A), pMLC (B) and pSTAT-5 (C). The effect of a cell permeable chemical inhibitor of ROCK (GSK429286A, 10µM) was determined on the phosphorylation of MLC (D). The time points at which the determinations were made are indicated in individual experiments.

A



IL-18: Healthy Vs Naïve $P < 0.05$ *

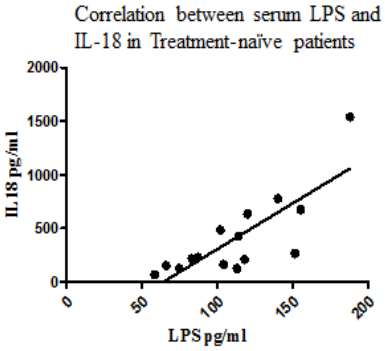
B



LPS: Healthy Vs Naïve $P < 0.05$ **

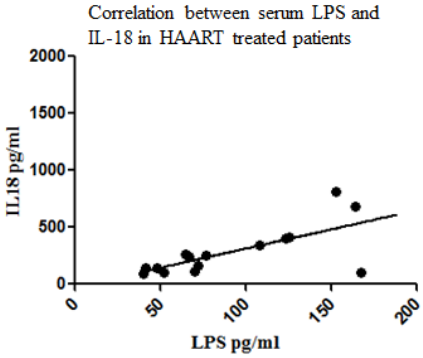
Elite Vs Naïve $P < 0.05$ *

C



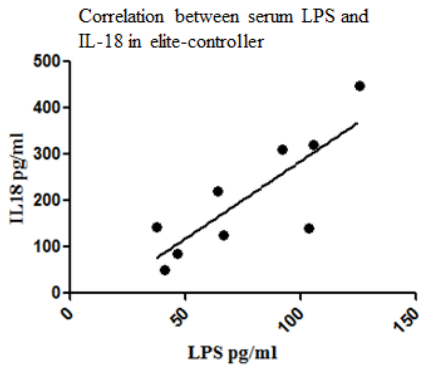
P value = 0.0004 $r^2 = 0.6359$

D



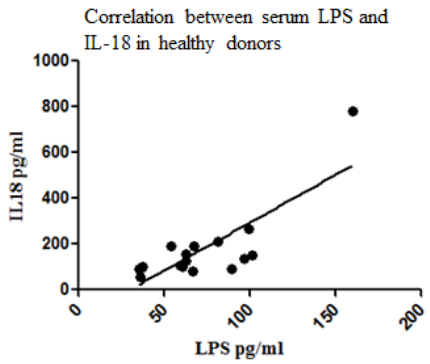
P value = 0.0041 $r^2 = 0.4824$

E



P value =0.0071 r^2 =0.665

F



P value < 0.0001 r^2 =0.6283

Figure 13

Figure 13. Correlation between serum IL-18 and LPS concentrations in HIV-infected and healthy control subjects.

Concentrations of serum IL-18 (A) and LPS (B) in different groups of HIV-infected individuals. The vertical lines show medians ± SD. The other panels show correlation between the two parameters in all HIV-infected (C), treatment-naïve HIV-infected (D), HARRT-treated HIV-infected (E), Elite-Controller (F), and HIV-seronegative healthy control individuals.

Table 1. Clinical parameters of HIV infected patients

Category	Number	CD4 Average	CD8 Average	Viral load in copies (log^{10}) per ml Average
Naive	15	551	744	3.5895
ART Treated	15	486	798	<1.7
Elite	9	826	502	Undetectable
Healthy	17	792	367	Not determined

In previous two chapters, two manuscripts based on our results have been presented. The following discussion, despite overlapping with the one presented in the manuscripts, is written in a wider context. It also provides a model for the role of IL-18 and IL-18BP based upon our findings and current literature.

Chapter 5

Discussion

1. Platelets produce IL-18 and release IL-18BP upon activation

IL-18 is gaining importance role in HIV infection. Our previously published research showed that IL-18 increases viral production in macrophages and T cells, and induces FasL expression in key innate immune cells, such as NK cells. Consequently, the cells may undergo apoptosis via Fas/FasL interactions [206]. This cytokine production is upregulated in the early stages of the infection and stimulates immune system activation. Potentially tissue destructive increases in IL-18 continue during HIV infection and promote AIDS progression. Furthermore, IL-18BP, the natural IL-18 antagonist, is reduced in HIV patients [222]. HIV infection initiates the production of IL-18 from many activated cells; however, this area of research has yet to be fully explored. Therefore, we were interested in studying a new source of IL-18 and IL-18BP in the human immune system and testing its importance during HIV infection. Platelets are considered an important source of cytokines, such as IL-1β, IL-7, and TGF-β, which are induced by different mechanisms. IL-1β is synthesized de novo upon platelet activation [102], while TGF-β is stored in them pre-made and is released from α-granules upon activation [264]. Our results show that platelets produce IL-18 upon activation. Interestingly, nonactivated platelets fixed by paraformaldehyde (PFA) did not express IL-18 in lysates or supernatants. In contrast, platelets contain pre-made IL-18BP and release it in to the supernatant irrespective of their activation. Platelets have the ability to continuously produce IL-18 upon activation, making them an important source of this cytokine in human plasma. Notably, precursor IL-18 and active IL-18 bands were not detected in Western blot assays using PFA-fixed platelets. Activated platelets release an abundance of active IL-18 in to the medium, which is dependent on the activator. The cytokine was not detected from PFA-fixed platelets medium or platelet lysates by using ELISA. However, IL-18BP was detected in both PFA-fixed and activated platelets in Western blots and ELISA assays. It is widely believed that platelets store many pre-made proteins obtained from megakaryocyte progenitor cells, and release them upon activation. The presence of larger quantities of IL-18BP in nonactivated platelet lysates supports the concept that platelets do not synthesize IL-BP but contain it in a pre-made form derived from megakaryocytes or surrounding milieu.

2. Platelets synthesize IL-18 *de novo* from the gene transcripts upon activation

Our finding that both precursor and mature IL-18 forms are absent from nonactivated platelets that were fixed by PFA immediately after blood collection [chapter 3, Figure 1A] suggests that the cytokine is produced de novo in platelets, and that IL-18 gene transcripts are present in platelets. We tested this possibility by RT-PCR. Interestingly, transcripts for IL-18 and GAPDH but not for IL-18BP or IL-8 were detected in platelets. We further confirmed our findings using cycloheximide, an inhibitor of translation [265]. The treatment of platelets with this inhibitor prevented expression of IL-18 but not of IL-18BP in platelets. Furthermore, PFA-fixed platelets did not stain for IL-18, while activated platelets showed clear staining for IL-18 that co-localized with F-actin. In contrast, both fixed and activated platelets showed staining for IL-18BP co-localizing with F-actin.

Platelets are small anucleate cells that lack genomic DNA but have been shown to cotain transcripts for a variety of genes. They retain the ability to splice these transcripts and synthesize proteins from these transcripts [266]. Platelets acquire approximately 6000 mRNAs species from megakaryocytes that are translated into proteins upon platelet activation [267]. It is known that this acquision is non-random. However, the exact rules governing the selection of these mRNAs species remain unknown. Each platelet contains approximately 2×10^{-15} g mRNA, making a total of 20 ng RNA from 10^7 platelets [268]. This amount of mRNA allows platelets to produce a select group of proteins de novo upon activation. Platelets synthesize GPIb, αIIbβ3, fibrinogen, thrombospondin, albumin, von Willebrand factor, various contractile proteins, HLA and coagulation factor XIIIa de novo [269, 270]. Interestingly, earlier reports demonstrated that platelets contain mRNA that encodes IL-1β in platelets polysomes [102, 271]. Activated platelets induce rapid and sustained synthesis of IL-1β de novo [102]. Our findings that IL-18 is another cytokine whose mRNA is present in platelets and they synthesize it *de novo* upon activation.

3. Production of mature IL-18 in platelets requires assembly of inflammasome and caspase-1 activation

IL-18 is a member of the IL-1 family and shares a number of characteristics with IL-1β. Both cytokines are produced as inactive precursors that need to be cleaved to form mature functional cytokines. This process requires caspase-1 activation by assembled inflammasomes. Inflammasomes are multiprotein complexes activated by so-called endogenous (eg, ATP, urea crystals, and contact allergens) or exogenous (eg, viruses, bacteria, and toxins) signals; reviewed in [123]). Inflammasome assembly results in the activation of caspase-1, needed to produce mature IL-18 from its precursor. Our co-immunoprecipitation results demonstrate that inflammasome assembly occurs in activated human platelets to produce mature IL-18 (Figure 3). We subsequently used glyburide to confirm this result. Glyburide is used in the United States as a treatment for type 2 diabetes [272]; it prevents caspase-1 activation and inflammasome assembly [273]. Macrophages that were pretreated with glyburide were then stimulated with ATP and LPS; this led to complete inhibition of IL-1β production and demonstrated the inhibitory effect of glyburide on inflammasomes [273]. We showed here that mature IL-18 production was inhibited in glyburide-pretreated platelets stimulated by thrombin. Our findings are in line with recent published findings that describe IL-1β production from human platelets after inflammasome assembly [274].

Since we expected inflammasome assembly to increase caspase-1 activity during platelet activation, we used a caspase-1/ICE colorimetric assay to measure caspase-1 activity in thrombin-activated platelets. As expected, high levels of active caspase-1 was detected following platelet activation. Pretreatment with a specific caspase-1 inhibitor or glyburide reversed this effect and prevented caspase-1 activation. This explains why we do not observe mature IL-18 in the supernatant of activated platelets pretreated with the caspase-1 inhibitor or with glyburide in our experiments. Our theoretical model of IL-18 and IL-18BP release from human platelets is demonstrated in **Figure 9** in this chapter.

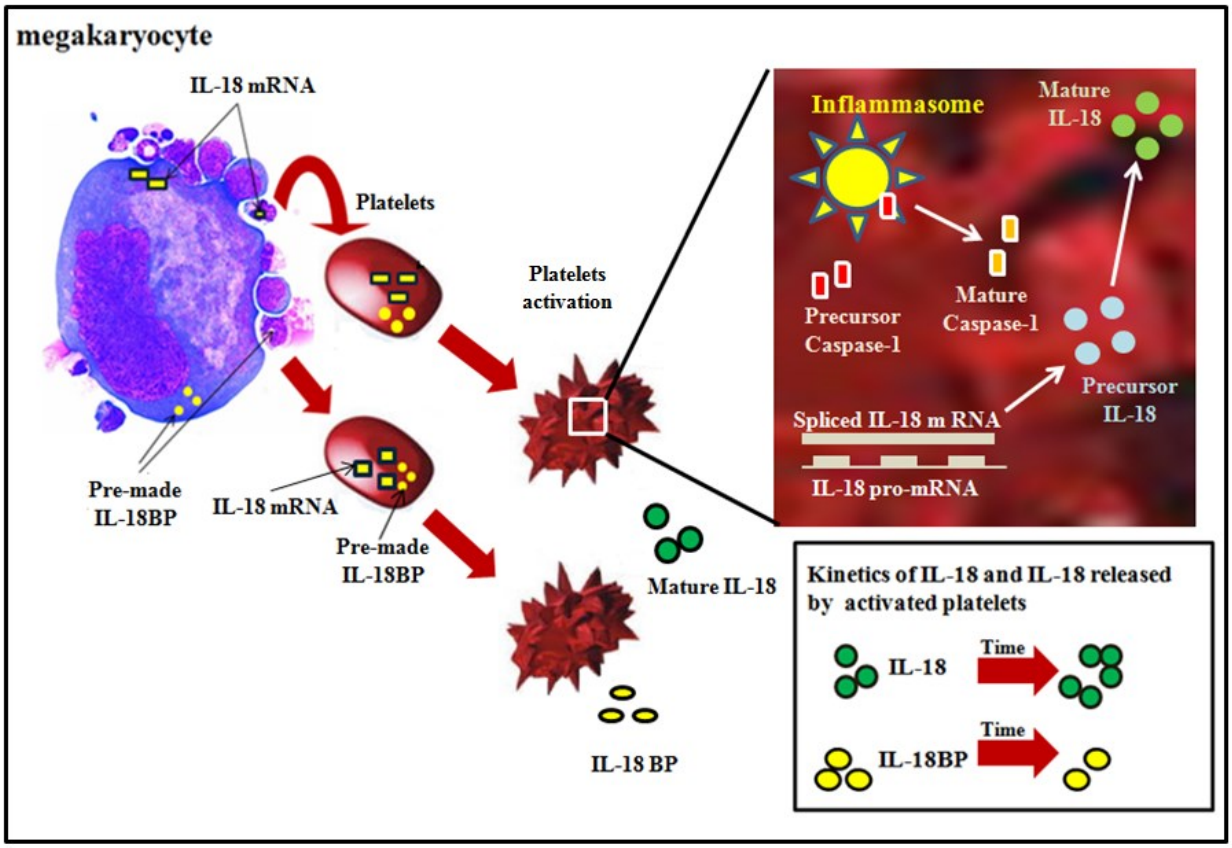


Figure 9. The theoretical model of IL-18 and IL-18BP release from human platelets.

Pre-made IL-18BP derived from megakaryocytes is stored in platelets and is released constitutively as well as upon activation. Platelets contain IL-18 transcripts that are used to synthesize precursor IL-18. Activation of platelets leads to inflammasome assembly and release of active caspase-1 that cleaves precursor IL-18 into mature IL-18. Mature IL-18 is then released in to the circulation. It is well known that activation of capase 1 and secretion of mature IL-18 in humans is coupled. The Figure also illustrates the kinetics of IL-18 and IL-18BP production in platelet supernatants over time.

4. Platelets contribute to IL-18 and IL-18BP in the human circulation in activation dependent and independent manner, respectively

The data from our first manuscript (chapter 3) shows that upon activation, platelets produce IL-18 *de novo*, process and release it. This finding prompted us to examine whether platelets contributed to IL-18 in the circulation. If so, then what was the role of their activation? As only activated platelets expressed and released IL-18, it was obvious that only activated platelets could have contributed to the concentrations of this cytokine in the circulation. As we found that freshly isolated platelet-poor-plasma (PPP) from healthy individuals lacked IL-18, and plasma samples from the same individual had significantly higher levels of this cytokine, we conclude that activated platelets are major contributors of this cytokine in these body fluids. As mentioned above, our results showed that IL-18 levels in PFA-fixed platelets and PPP were significantly lower than in the plasma or serum from the same individuals. However, we did not detect a significant difference in IL-18BP levels between PPP and plasma. Furthermore, we observed that whether activated or not, platelets express IL-18BP and release it into the medium. In fact the amount of the antagonist expressed in platelets and released into the medium decreases upon activation [chapter 3, Figure 8A, C, and D]. Taken together these observations suggest that platelets contribute to the circulationg levels of this soluble mediator irrespective of activation. Their contribution, in fact, may decrease when they are in activated state. As many other cell types in the body produce IL-18BP, certainly, they may also be contributing to its levels in the circulation. Here we were not interested in studying the comparison between platelets vs other cell types with respect to their contributions to IL-18BP in the circulation. We just wanted to emphasize that platelets are a novel source of this soluble mediator in the body. Other than IL-18 and IL-18BP, platelets are an important source of several circulating cytokines or chemokines [275-277], and are the main source of CD40 ligand (CD40L) in plasma [278, 279]. Moreover, decreased platelet counts in aplastic anemia and a mouse model of immune thrombocytopenic purpura is associated with decrease levels of chemokines, such as chemokine (C-X-C motif) ligand 5 (CXCL5), chemokine (C-C motif) ligand 5 (CCL5 or RANTES),

epidermal growth factor (EGF), and CD40L [280]. Whether levels of IL-18 and IL-18BP decrease in these conditions is not known.

Plasma concentrations of certain proteins released by platelets, such as IL-7, RANTES, beta-thromboglobulin (beta-TG), and platelet factor 4 (PF4), increase after 5 or 7 days of platelet storage [281]. This prompted us to investigate the kinetics of IL18 and IL-18BP release by platelets. Our kinetics results are in agreement with these findings at least for IL-18. We found that the release of this cytokine from activated platelets continued to increase overtime. In contrast, IL-18BP secretion remained at a stable level over 24 hours. Unexpectedly, IL-18BP levels decreased following one hour of platelet activation. Rapid degradation of cytokines, such as TNF-α, after one hour at room temperature have been reported [282]. Further studies are required to understand the mechanism(s) behind this decrease. We speculate that platelet activation induces *de novo* synthesis and/or activation of one or more proteases that cleave IL-18BP.

5. Implications for HIV-infected individuals

This research work highlights the important role of platelets to produce IL-18 in humans. As previously described, IL-18 released from platelets contributes to IL-18 concentrations in the circulation in healthy individuals [260]. Platelet activation increases the production of this cytokine as detected in platelet supernatants. Interestingly, our results also show significant increases in IL-18 levels in PPP from HIV infected patients compared with healthy individuals. This suggests that increased amount of the cytokine is being produced in the body of HIV-infected individuals. As platelet activation has been associated with HIV infection [283], and appears to be induced largely by HIV-1 Tat protein [95], the viral protein seems to be responsible for enhanced production of this cytokine from platelets. We also demonstrated that LPS activates platelets and increases plasma IL-18 (Figure 1B and Figure 1C). Elevated LPS in patients with HIV could activate IL-18 synthesis in the circulation [284].

Increased caspase-1 gene expression was previously observed in HIV-infected cell lines as well as in the T cells of patients infected with HIV [285]. In addition, inflammasome

activation and increased IL-18 production were demonstrated recently in cultured HIV-infected monocytes [286]. When HIV infects the THP-1 cell line, it induces the assembly of NLRP3-ASC-caspase-1 complex and, as a consequence, increases IL-1β production [287]. The synthesis and secretion of IL-1β after activation of NLRP3 inflammasome in the platelets of Dengue virus-infected patients was also recently described [274]. In the present thesis, we described platelets as a novel source of IL-18 in the circulation. Since IL-18 levels were elevated in PPP from HIV-infected patients but not from healthy ones, and there was no corresponding increase in IL-18BP in the PPP of the patients, our results suggest that PPP could serve as an appropriate biological specimen to estimate imbalance between IL-18 and its antagonist in HIV-infection and possibly in other chronic viral infections. In this regard, a differential induction of the two soluble mediators by HIV-1 from human macrophages was reported earlier from this laboratory [222]. This imbalance contributes to immunopathogenesis of HIV disease in multiple ways (reviewed in [206]). In addition, the positive correlation between elevated IL-18 in HIV infection and platelet activation was described by our laboratory [260]. Although, the role of platelets in HIV pathogenesis is not fully elucidated, our studies provide new evidence for their role in HIV infection.

6. HIV infection increases IL-18 and decreases IL-18BP production *in vitro* from IEC

While much research and clinical data have demonstrated that HIV-1 infection is associated with increased intestinal permeability, the mechanisms underlying HIV-induced disruption of the mucosal barrier are not fully understood. Here, we showed that intestinal epithelial cells exposed to HIV-1 or its viral protein Tat, upregulated IL-18 and downregulated IL-18BP production. This effect was demonstrated both in cell lysates and supernatants (Chapter 4, manuscript 2, Figure 1 and 3). Interestingly, incubating HT29 cells with the mock viral preparation or Tat neutralized with an anti-Tat antibody did not produce increases in IL-18 or decreases in IL-18BP in supernatant (Chapter 4, manuscript 2, figure 3). These findings suggest that upregulation of IL-18 from HT29 cells is specific to HIV-1 and its protein Tat.

Recently, Kaushic's research group proposed that epithelial cells are the primary source of inflammatory cytokines that disrupt the intestinal mucosal barrier [49]. The authors were able to demonstrate that incubating HIV with the T84 intestinal cell line led to enhanced production of a number of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 [49]. Our current results show in another IEC line (HT29) that the virus also induces production of IL-18, and reduces that of its antagonist. It appears that the virus promotes inflammatory milieu after its interaction with the IEC. The caveat here is that Caco2 and HT29 are tumor cell lines, and do not represent primary intestinal epithelial cells. Therefore, these results should be validated using primary intestinal epithelial cell cultures. Our laboratory has previously published an *in vitro* study demonstrating that IL-18 production from human monocyte-derived macrophages is upregulated after HIV infection [222]. The ability of HIV to increase IL-18 production in humans was supported by clinical data that described significant associations between increased IL-18 serum levels with HIV seropositivity as well as with AIDS-associated events [288].

In experiments designed to determine whether HIV-induced IL-18 release is dependent upon caspase-1, we found decreased IL-18 production in the lysate of HT29 cells pretreated with a caspase-1 inhibitor before infection with NL4.3 HIV. This was associated with increased precursor IL-18 bands and decreased mature IL-18 bands in western blot assays. Interestingly, caspase-1 inhibitor has been shown to inhibit HIV-mediated cell death by pyroptosis in CD4+ T cells [13]. We show here that this treatment also prevents HIV-induced processing of precursor IL-18, and release of mature IL-18 from platelets and IEC.

7. IL-18 induces intestinal cell death in a concentration- and time-dependent manner

It has been reported that proinflammatory cytokines induce intestinal cell apoptosis resulting in the formation of large paracellular gaps or openings between cells [289, 290]. Therefore, we examined the possibility that IL-18 induces HT29 cell apoptosis. We previously reported the apoptotic effect of IL-18 in NK cells, which were indirect and were mediated via Fas/FasL interactions [291]. In the present thesis, our results show that IL-18 caused progressive

time-dependent cell death in IEC monolayers. We also examined the effect of different IL-18 concentrations. Interestingly, our data revealed that IL-18 causes epithelial cell death in a concentration-dependent manner. More importantly, neutralizing recombinant IL-18 with an anti–IL-18 antibody prevented IL-18–induced apoptosis and proved that the effect was specific to the cytokine itself. The pro-apoptotic effects of this cytokine have also been reported for cardiac and microvasculature endothelial cells [292].

It is important to note that different reports describe apoptosis as being induced by different cytokines in IECs. Morphological changes and cell apoptosis were induced after incubating HT29 cell lines with TNF-α [293]. Additionally, IL-13 induced apoptosis in intestinal cells and this effect was associated with increased intestinal permeability [294].

8. IL-18-induced cell death involves caspase-1 and caspase-3 activation

To confirm that HT29-induced cell death was mediated by apoptosis and not by necrosis, we performed assays to detect the presence of caspase-1 and caspase-3 in cell lysates after IL-18 treatment. LPS treatment was chosen as a control because it is known to increase both caspase-1 and caspase-3 activity [295]. Our results revealed increased expression for active caspase-1 and caspase-3 in cells treated with LPS or with IL-18 compared to untreated cells. This finding was in line with recent published reports describing IL-18–induced increases in caspase-3 in renal tubular cells [205]

To know whether the activation of caspase-1 and caspase-3 is essential for IL-18–induced apoptosis, we pretreated cells with a specific caspase-1 inhibitor, caspase-3 inhibitor, or both the caspase inhibitors together. As we expected, caspase-1 and caspase-3 inhibitors significantly reduced IL-18–induced apoptosis. However, pretreatment with both the inhibitors combined did not show any additive effect. As previously mentioned, TNF-α and IL-13 induce intestinal cell apoptosis. Interestingly, IL-13–induced apoptosis is inhibited by 50% when the cells are pretreated with the pan-caspase inhibitor Z-VAD-FMK [296]. Furthermore, caspase-3

and caspase-1 inhibitors completely inhibit TNF-α/INF-γ–induced apoptosis in human intestinal epithelial crypts [297]. It is quite possible that some caspases other than capase-1 and caspase-3 may also be involved in IL-18-induced apoptosis of IEC. In this regard, it would be interesting to see the effects of a pan-caspase inhibitor. It is important to mention that, although IL-1β shares many characteristics with IL-18, including disruption of the intestinal barrier, epithelial cell death by apoptosis remains specific to IL-18, as IL-1β does not induce apoptosis in IEC [69, 71].

Cytokines have been shown to increase intestinal permeability by modulating tight junction proteins, such as claudin-2, claudin-4, and occludin [298], by modulating adherent junction proteins, such as β-catenin [299], and by affecting cytoskeleton actin filaments (F-actin) [63, 300]. It is interesting to note that rearrangement of F-actin could induce disruption in tight Junction assembly [301]. Changes in F-actin dynamics were reported as a mechanism of intestinal epithelial barrier leakage by TNF-α [300]. Therefore, we investigated whether IL-18 induced changes in the expression of tight and adherens junction proteins.

In line with published literature concerning the mechanisms of intestinal barrier disruption by proinflammatory cytokines, we found that IL-18 rearranged β-catenin distribution in HT29 cells after 24 hours of treatment (Chapter 4, manuscript 2, Figure 7). Furthermore, decreases in claudin-2 and occludin were detected in Caco2 and HT29 cells, respectively after IL-18 treatment (Chapter 4, manuscript 2, Figure 8). According to the literature, TNF-α also disrupts barrier function in T-84 intestinal cells [298]. The HIV-1 Tat protein was used as a control in these experiments because of its ability to alter the integrity of tight junctions in epithelial cells [302]. We further examined whether IL-18 induced changes in mucosal permeability by causing structural alterations of the F-actin cytoskeleton. Our findings from both Caco2 and HT29 cell lines revealed as significant reduction in the intensity of F-actin with extensive disorganization. Interestingly, cells treated with IL-18 and Tat in combination exhibited a potentiated decrease in F-actin intensity and rearrangement. This synergistic effect could be explained by increased caspase-3 activity. Various reports have suggested that activation of caspase-3 decreases F-actin and causes its collapse [303]. It has been reported that increased apoptosis occurs via actived caspase-3 after Caco2 cells are treated with Tat [51]. As

our results show that IL-18 activates caspase-3 in IEC, the activated caspase may cause desctruction of F-actin in these cells. A model of how IL-18–induces increase in intestinal permeability during HIV infection is proposed in (**Figure 10** in this chapter).

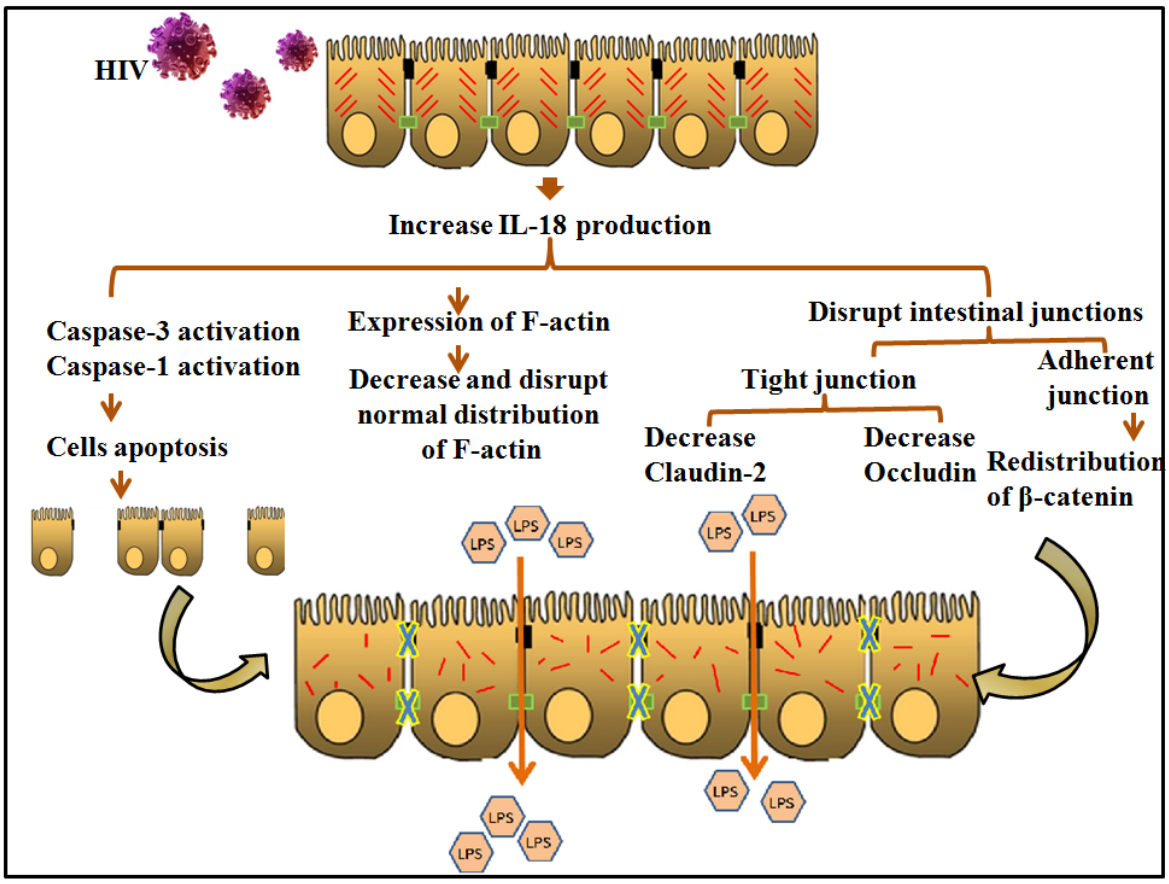


Figure 10: Proposed model of IL-18–induced intestinal permeability by HIV infection. IL-18 disrupts intestinal tight junctions, and increases intestinal cell apoptosis by caspase-1 and caspase-3 activation. The cell apoptosis creates gaps between cells that further increase permeability. By affecting F-actin, β-catenin, occludin, and claudin-2 distribution, IL-18 disrupts not only tight junctions but also adherent junctions. Together, these properties of IL-18 lead to increased permeability and translocation of bacterial products, such as LPS.

The IL-18-induced changes in tight and adherens junction proteins mentioned above prompted us to directly examine the effects of this cytokine on intestinal permeability. High mucosal permeability can be detected with TEER and Lucifer Yellow flux [304]. Therefore, we used TEER to examine the effect of IL-18 on paracellular permeability. We performed TEER experiments using the Caco2 cell line, as the cell monolayers show higher electrical resistance ranging between 1,400 and 2,400 Ω cm^2 compared with HT29 cells, which have electrical resistances around 100 Ω cm^2 [305]. Therefore, Caco2 cells are usually used for measuring the effects of different substances on TEER. As expected, IL-18 and Tat increased paracellular permeability and decreased TEER. Tat-induced decreases in TEER were previously shown in human retinal pigment cell line D407 [302]. We used IL-1β as a positive control, as it is known to decrease TEER in epithelial cells. Concurrent with previous research, IL-1β induced a transient increase followed by a sharp decrease in TEER after 10 hours [68].

To determine how IL-18–induced epithelial junction disruption could affect molecular traffic crossing the intestinal cell monolayer, we used the fluorescent tracer Lucifer Yellow to determine transepithelial transport through paracelluar junctions after treating cells apically or basolaterally with the cytokine. Lucifer Yellow is a dye with a very small molecular weight (0.45 kDa) that has been frequently used for measuring intestinal permeability [306]. Our results showed that IL-18 increased Lucifer Yellow transport when applied to the apical surface of the cells while Tat increased the transportation when applied to both apical and basolateral surfaces. However, IL-1β increased permeability (measured by fluorescein sodium salt, 0.4kDa) when it was applied from basolateral to apical direction but not from apical to basolateral one. This finding was in line with a published report [68]. It is not known why IL-18 affects intestinal permeability when applied to the apical surface of the monolayers, and not when applied from the basolateral surface. It is quite possible that the cytokine receptors are expressed on the apical surface. Further studies are required to test this hypothesis.

9. IL-18 increases expression of MLCK and induces MLC phosphorylation via ROCK

Recent studies have shown that cytokines, such as TNF-α and INF-γ, affect TJ permeability by increasing expression of MLCK and consequently increased MLC phosphorylation [300, 307]. Activation of MLCK disrupts the epithelial barrier and increases bacterial translocation [308]. Increases in phosphorylated MLC cause rearrangement in F-actin and disrupt tight junction assembly [301]. However, various kinases, such as ROCK, protein kinase C, and MLCK have been reported to regulate MLC phosphorylation [300]. Here, we show that IL-18 upregulated MLCK and MLC phosphorylation after 10 and 30 minutes of the cytokine treatment. Interestingly, we also show the synergistic effect of HIV-1 Tat and IL-18 in phosphorylating MLC after one hour (Chapter 4, manuscript 2, Figure 12B). As MLCK-mediated MLC phosphorylation is dependent on ROCK. We suspected that ROCK may also be involved in the IL-18 induced increased phosphorylation of MLC. As expected, cells treated with a ROCK inhibitor did not show increases in MLC phosphorylation after incubation with IL-18 for 30 minutes. Together, these data suggest that IL-18 uses the same signaling pathway as used by TNF-α for disrupting the integrity of intestinal epithelial cell monolayers [309, 310].

The data from this study provide a specific signaling pathway that may be involved in IL-18–mediated increases in intestinal permeability, and we have illustrated this in **Figure 11.** We demonstrated here for the first time that IL-18 activates caspase 1 and caspase-3 in IECs. Interestingly, it has been reported that MLCK activity may be directly enhanced by activated caspase-3 [311]. Furthermore, it was reported that a specific caspase-3 inhibitor reduces changes in tight junctions induced by *Giardia lamblia* in the human duodenal epithelial cell line SCBN [312]. It was also described that caspase-3 constantly activates ROCK leading to regulated actin–myosin cellular contraction in HeLa cells [313, 314]. In addition, ROCK was also reported to induce MLC phosphorylation leading to cytoskeletal reorganization in intestinal cells [315]. Our results are in accordance with these earlier reports.

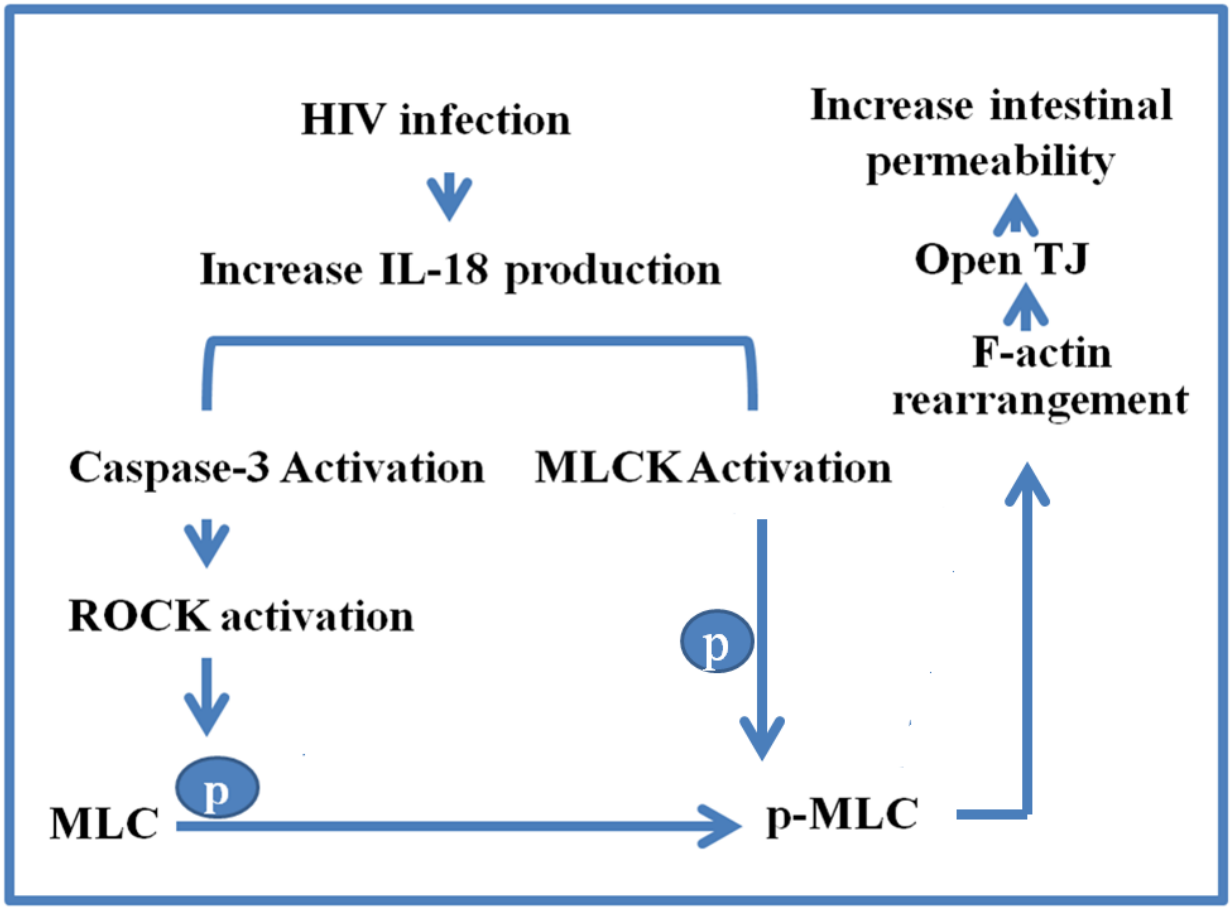


Figure 11: Molecular mechanisms of increased intestinal permeability in HIV infection. HIV induces increases in IL-18 production that leads to activated MLCK and MLC phosphorylation. In turn, this compromises physiological functions of tight junctions resulting in increased intestinal permeability to microbial products from intestinal flora.

10. IL-18 concentrations correlate with LPS levels in healthy and in HIV-infected individuals

Increased intestinal permeability results in increased translocation of bacterial products and small bacterial fragments into body tissues and the general circulation [316]. Microbial products like LPS cause a generalized activation of the immune system, which is invariably observed in HIV-infected individuals [18]. LPS is a component of Gram-negative bacteria cell walls and considered a major marker of microbial translocation [317]. Correlations between increased LPS and elevated proinflammatory cytokines levels such as TNF-α, IL-1 and IL-6 were demonstrated in sepsis [318]. Also, LPS levels are positively correlated with IL-6 and IL-8 plasma levels in visceral leishmaniasis patients [319]. Furthermore, the correlation between LPS and INF-γ in HIV infection was also previously reported [18].

Our results reveal a significant positive correlation between increased LPS and elevated IL-18 in HIV-infected patients. Surprisingly, the same positive correlation was detected in healthy individuals. These data highlight the important role of IL-18 in intestinal permeability associated with HIV. Although plasma levels of proinflammatory cytokines, such as IL-1β, TNF-α, IL-18 and IL-6 were increased during HIV infection, and the role of cytokines in inducing intestinal permeability is well described, we showed here for the first time a positive correlation between increased translocation of bacterial products and elevation of IL-18 in HIV infection. It is noteworthy that a salient feature of IL-18 is to induce IFN-γ from several human cell types [320, 321]. Increased IL-18 concentrations may also result in increased concentrations of this interferon, known to cause disruption of intestinal epithelial barriers and hence correlation with LPS in the circulation [299].

This positive correlation between LPS and IL-18 in HIV infection provides important information for the understanding of HIV enteropathy and disruption of mucosal epithelial barrier and consequent immune activation. Because our research model shows that HIV infection increased IL-18 production from IECs, increased levels of this cytokine disturb the epithelial barrier by cellular apoptosis and paracellular (altered junction composition) changes, and leads to increased LPS translocation. Interestingly, LPS itself may have a disruptive effect on intestinal epithelial cells. For instance, in Caco2 cells, LPS increases intestinal tight junction

permeability [322]. In HIV infection, elevated LPS induces the production of more intestinal proinflammatory cytokines by activating the immune system [323].

HIV infection induces several changes in the profile of inflammatory cytokines including IL-18. We show here that IECs and activated platelets represent important sources of this cytokine. The increased concentrations of the cytokine disrupt intestinal barrier function, and cause cell death and enteropathy. Increased intestinal permeability results in translocation of microbial products like LPS and bacterial fragments into tissues and general circulation in HIV-infected individuals. Microbial translocation leads to aberrant activation of the immune system as well as to chronic low-grade inflammation. Thus IL-18 may represent an important molecular target in HIV infection.

Conclusions and future studies

A general conclusion from our study is that human platelets produce and secrete IL-18 and IL-18BP upon activation. Platelets also contain components of the inflammasome, which activate caspase-1 and process the precursor IL-18 into its mature form during the platelet activation process. Different agents activate platelets and release IL-18 to variable extents, the maximum effect being observed with thrombin. Contrary to IL-18, platelets constitutively express pre-formed IL-18BP, and release it irrespective of activation. Cycloheximide does not affect expression of IL-18BP in these cells. Platelet-released IL-18 constitutes the main source of this cytokine in the human circulation. We found decreased amounts of this cytokine in the platelet lysates of HIV-infected individuals as compared to healthy individuals. In contrast, its concentrations were increased in the serum and platelet-poor plasma in infected individuals. Similar findings were obtained with respect to IL-18BP in platelet lysates from healthy and HIV-infected individuals. However, lower amounts of this IL-18 antagonist were found in the serum and PPP of HIV-infected individuals compared with healthy individuals. Our findings signify the role of platelets in the dysregulated production of IL-18 and its antagonist in HIV-infection. Platelets also have important implications for other chronic inflammatory disease conditions in which increased IL-18 activity plays a pathogenic role.

In addition, we concluded that HIV infection increases IL-18 production from intestinal epithelial cells; this affects intestinal permeability and increases microbial translocation. We demonstrated that incubation of HIV with HT29 induces IL-18 and reduces IL-18BP production. We tested the effect of IL-18 in both HT29 and Caco2 intestinal cell lines. Interestingly, we noticed very high destructive effects on HT29 cell culture as compared with non–IL-18 treated cells. The cytokine was found to induce death via apoptosis in both HT29 and Caco2 cells, which was dose dependent. Furthermore, higher levels of caspase-1 and caspase-3 activation were detected by Western blot in HT29 cells' lysates after treatment with IL-18. This increase in apoptosis was associated with a concomitant decrease in intestinal tight junction proteins, occludin and claudin 2. IL-18 decreased adherent junction-associated proteins like beta-catenin and disturbed the spatial arrangement of F-actin in Caco2 and HT29 cells. Caco2 cells showed

a decrease in *TEER* after 24-h treatment with IL-18. We also investigated concentrations of LPS and IL-18 in the circulation of HIV-infected and HIV-seronegative healthy individuals. Interestingly, we found significant positive correlations between these two parameters in different HIV-infected individuals as well as in healthy donors.

Overall, our results have important implications for HIV-induced AIDS and AIDS-related clinical conditions. They are also relevant to other chronic viral infections in which platelets become activated *in vivo* and increase IL-18 concentrations. We demonstrated that HIV-induced IL-18 production from intestinal epithelial cells. IL-18 plays a role in microbial translocation and chronic immune activation in HIV-infected individuals.

Future studies should be conducted along the following lines:

1. It should be investigated why platelet activation results in decreased production of IL-18BP, whether exogenous IL-18BP could neutralize and overcome the pathogenic effects of the cytokine on IEC.
2. It is amply clear now that platelets contain a repertoire of cytokines and growth factors with diverse and often opposite immunological and biological effects. It should be worth investigating the stimuli that promote secretion of one or the other type of these factors. For example identifying the stimuli that induce release of anti-inflammatory cytokines may help in reducing inflammation in HIV infection and other human diseases.
3. It is noteworthy that increased concentrations of IL-18 and decreased/or non-elevated concentrations of its antagonist in HIV infection are based upon *ex vivo* studies using biological samples from HIV-infected individuals. It would be important to investigate the expression of these two soluble mediators locally in the intestinal, skin and vaginal tissues from patients.
4. We have investigated the effects of IL-18 on intestinal integrity and barrier function using established IEC. These IEC are not normal and are derived from intestinal cancers. It is important to verify these findings should be verified in the primary intestinal epithelial cells and ideally in organ cultures using intestinal biopsies.
5. Recently, a new player, IL-37, has been identified that regulates IL-18/IL-18BP system. Belonging to the IL-1Family, it exerts anti-inflammatory effects. It binds IL-18BP and

increases its ability to neutralize IL-18. Virtually nothing is known on how it is regulated in HIV infections, and how it affects pro-inflammatory effects of IL-18.

6. It will be important to know how increased concentarions of IL-18 affect anti-viral adaptive immunity. Although the cytokine is known to promote Th1 type responses, it does so by inducing IFN- γ from NK and T cells. However, IL-18 alone is ineffective and cannot induce this interferon without collaboration from other cytokines notably IL-12. It still remains unknown how it would affect anti-HIV responses given that IL-12 production becomes compromised in this viral infection.
7. Finally the effects of neutralizing IL-18 on HIV pathology and immunity should be studied in an appropriate animal model of HIV.

These proposed investigation may provide a clear picture as to whether and how IL-18/IL-18BP system could be targeted to delay AIDS progession and augment anti-viral immunity in HIV infections.

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